LOSS OF P120CTN: ITS EFFECT ON CADHERIN LEVELS, DEVELOPMENT, AND TUMOR PROGRESSION

By

Michael Alan Davis

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

May, 2005

Nashville, Tennessee

Approved:

Albert B. Reynolds, Ph.D

Lynn M. M. Matrisian, Ph.D

Christopher Wright, Ph.D.

Josiane Eid, Ph.D.

Carlos Arteaga, M.D.

Robert Whitehead, Ph.D.

This work is dedicated to all those who have accompanied me along this journey… And there have been so many! You have trained me and guided me. You have kept me company and picked me up when I was down. Without you this journey would have been impossible. For that I thank you with every cell of my being.

ACKNOWLDGEMENTS

There are so many…

First, I would like to thank my PI, Al Reynolds. I came to this lab looking for a mentor, and you have filled that role in every sense of the word. You gave me the freedom I needed yet were always there when I needed you. Through your tutelage, I have become the scientist I am today. I have learned much from you Al, and I can not thank you enough. I pray that we always keep in touch, as your lab family grows over the years to come.

Next I would like to thank my thesis committee: Christopher Wright, Lynn Matrisian, Carlos Arteaga, Robert Whitehead, and Josiane Eid. You, also, have been exactly what I needed. You are leaders in your fields. You provided invaluable aid and insight as I wove my way through the barriers of this project. You enhanced my work. You put up with my questions and took the time to listen. Thank you.

Lab mates, you probably deserve the most credit, or at least the most sympathy. You dealt with me on a daily basis. When I needed to pester someone, you graciously put up with it. If I had a question, you would try to answer it. You provided distractions from the frustrations, and you were there to celebrate with me as I passed scholastic and experimental milestones. I have been here longer than I had hoped, and I have seen you come and go. Together, we have become a family. Thank you. …and especially you, Pam. Thank you.

My work is the first animal research in the lab. There was no one in the lab from which to learn all the techniques required to complete my work. As a result, I became a

child of the department… or rather; I imposed myself on the department. From the Matrisian lab I thank Howard Crawford, Barbara Fingleton, Lisa McCawley, and Mark Gustavson. From the Moses lab, I thank Anna Chytil and Brian Bierre. I would like to think members of the Arteaga lab and Wright lab. I would like to thank David Threadgill and Scott Pearsol for their help in designing the targeting construct. Thanks go to Maureen Gannon and Laura Wilding for help with embryonic dissections and beta-galactosidase staining, respectively. I know that there are so many more within the department who have helped me in small ways over the years. Please know that you are not forgotten. You are all much appreciated. I could not have done this work without you.

We are blessed at Vanderbilt by so many core labs. The specialization and division of labor you provide greatly increases the speed of our science. Also, you provide an invaluable source of knowledge from which to draw. Specifically, I would like to thank Cathy Pettepher and the ES Core Lab for help and patience in generating the mice. I would like to thank Tony Frasier of the Human Tissue Acquisition Core lab with expert aid and advice in tissue processing and sectioning. You saved me so much time! I would like to thank members of the Animal Care staff, and I would like to thank Jay Jerome and Dorinda Vardell of the Electron Microscopy Core lab. Finally, I want to thank Khalari Bhat. You helped my through 20 kb of genomic sequence. Had I began a year later, I never would have needed to sequence so much DNA, but I never would have had the pleasure of knowing you. Thank you all.

Peter Dempsey, thank you for your help early in my graduate career and thank you for suggesting Al's lab.

Kay Washington, you have been so kind. I needed a crash course in histology. You patiently provided it. Your opinions and advice on perceived phenotypes was invaluable. You were fun to work with.

Throughout my graduate career, family and friends have supported me. I have met amazing people and shared many incredible experiences. Thank you.

Thank you Mom and Dad. You have always encouraged me. You have always supported me. You are my biggest fans. Thank you.

Christopher Wright. I know that I have mentioned you already, but thank you for going above and beyond the call of duty. It has been a pleasure working with you and getting to know you. Best of luck to you.

My friends, thank you. Thank you for letting me be me. Thank you for your acceptance. Thank you for the times we have shared. Thank you for the laughter and tears. Thank you for being part of my life and part of my experience here at Vanderbilt--you did not have to, yet you did. Especially you Chris "Topher" Sansam. Thank you.

Finally, thank you Reneé. I wish I had the words to adequately sing your praises. I hope you know that I love you and that I appreciate you. You are my best friend. You are my partner and collaborator. Having you by my side, I eagerly anticipate the future to come. Thank you.

Thank you all…

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LIST OFABBREVIATIONS

APC	Adenomatous Polyposis Coli
ARM	Armadillo
ARVCF	Armadillo repeat gene deleted in Velo-Cardio-Facial syndrome
BAC	Bacterial artificial chromosome
bp	Base pairs
BrdU	Bromo-deoxyuridine
C-cadherin	Xenopus E-cadherin
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
Crb3	Crumbs 3
C-terminus	Carboxy-terminus
ctn	Catenin
DN	Dominant negative
DNA	Deoxyribonucleic acid
dpp	Days postpartum
e14.5	Embryonic day 14.5
E-cadherin	Epithelial
EGF	Epidermal growth factor
EGFR	EGF Receptor
EMT	Epithelial to mesenchymal transition
ES cells	Embryonic stem cells

FACS	Fluorescent-activated cell sorting
GFP	Green fluorescent protein
GSK3	Glycogen-synthase kinase 3-beta
GTP	Guanosine triphosphate
hnRNA	Hetero nuclear RNA
hrs	Hours
h siRNA	Human p120-specific siRNA
HUAEC	Human umbilical arterial endothelial cells
IGFR	Insulin-like growth factor receptor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
keV	kiloelectron volts
kB	Kilobases
LSB	Laemmli sample buffer
LZRS	Lazarus
g	Microgram
M	Micromolar
mAb	Monoclonal antibody
mg	Miligram
mL	Mililiter
mm	Milimeter
mM	Milimolar
MMTV	Mouse mammary tumor virus

mRNA	Messenger RNA
m siRNA	Murine p120-specifid siRNA
N-cadherin	Neuronal-cadherin
Neo	Neomycin resistance gene
nM	Nanomolar
N-terminus	Amino-terminus
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PAS-stain	periodic acid Schiff stain
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
RT-PCR	Reverse transcriptase PCR
RNA	Ribonucleic acid
P-cadherin	Placental-cadherin
pRS	pRetro-SUPER
SDS	Sodium Dodecyl Sulfate
SH2	Src homology domain 2
siRNA	small interfering RNA
Tcf	T-cell factor
TK	Thymidine kinase gene
TUNEL	Terminal deoxynucleotidyl transferase-mediated end labeling
VE-cadherin	Vascular endothelial cadherin

CHAPTER I

INTRODUCTION

An Introduction to p120

p120ctn (p120 hereafter) was first discovered as a substrate for the src tyrosine kinase (Reynolds et al., 1992), and it is the prototypic member of the p120-subfamily of proteins that includes p0071, ARVCF, and δ-catenin(Anastasiadis and Reynolds, 2000). Like p0071 and ARVCF, p120 binds the juxtamembrane domain of E-cadherin where it likely regulates cadherin function (Ireton et al., 2002a; Thoreson et al., 2000; Yap et al., 1998). Furthermore, there is a strong correlation between p120 loss in tumors and loss of E-cadherin, a known tumor suppressor (reviewed in (Thoreson and Reynolds, 2002)). Together, these observations suggest that p120 may have a role in tumorigenesis and/or metastasis, however there is no direct evidence showing a role for p120 in tumor progression.

p120 Structure-Function

The p120 gene spans 50-60 kB and is subject to extensive alternative splicing (Keirsebilck et al., 1998). It contains 21 exons, four translational start sites, and three alternatively spliced exons. The extensive splicing gives rise to 32 theoretical p120 isoforms (Figure 1), but only a fraction of these are observed. p120 isoforms 1 and 3, which arise from the first and third translational start sites respectively, are the most prevalent and the best characterized. While both are usually expressed in any given cell,

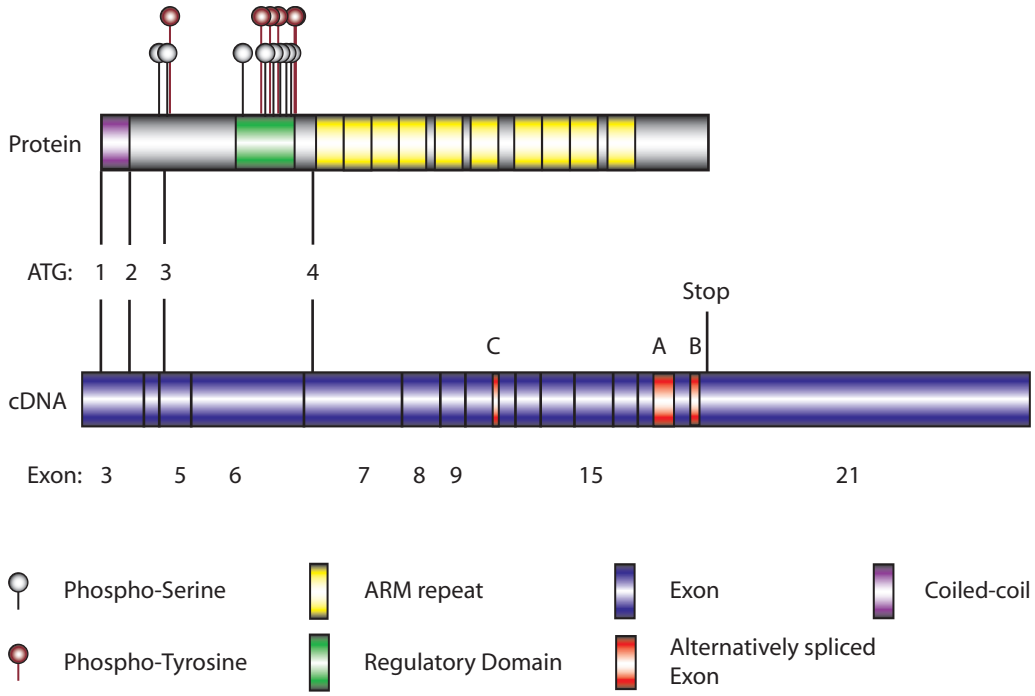


Figure 1. Functional domains of p120 Protein and Organization of p120 cDNA. p120 is a phosphoprotein that is phosphorylated on serine, threonine, and tyrosine. Most of these sites localize to an area named the phospho-regulatory domain, which is implicated in the negative regulation of cadherin function. p120 has 10 central ARM domains and an N-terminal coiled-coil domain. Both of these types of domains mediate protein-protein interactions, and it is likely that p120 is an adaptor protein that recruits regulatory proteins to the cadherin complex. At the DNA level, p120 has 21 exons and four translational start sites (ATG's 1-4) and 3 alternative exons (A-C) that arise through alternative splicing.

isoform 1 is predominantly found in migratory cells, such as fibroblasts and epithelial cells that have undergone EMT, and isoform 3 is primarily expressed in epithelial cells (Aho et al., 1999; Mo and Reynolds, 1996; Tran et al., 1999). We know little regarding the expression profiles and function of each specific isoform, however the size of the gene and the degree of splicing must be considered when developing conditional knockout targeting constructs.

The protein generated from the p120 gene contains three well-characterized domains (reviewed in (Anastasiadis and Reynolds, 2000)): an N-terminal coiled-coil, a phospho-regulatory domain, and an ARM domain. The coiled-coil domain is located at the extreme N-terminus of the p120 protein and is found only in p120 isoform 1. Coiled-coil domains typically mediate protein-protein interactions, but no proteins have yet been found to interact through this domain. Thus its function remains elusive.

p120 is phosphorylated on serine, threonine, and tyrosine (Mariner et al., 2001; Xia et al., 2003). The major phosphorylation sites have been mapped and cluster in an N-terminal region dubbed the phospho-regulatory domain. This domain appears to dampen the role p120 plays in mediating E-cadherin function, as isoform 4, which lacks the entire N-terminus, is better able to restore defective epithelial morphology in SW48 cells than isoforms 1 and 3 (Ireton et al., 2002a). However, the precise role of each phosphorylation site is unknown.

p120 is part of a larger family of ARM domain proteins that includes β-catenin (Daniel and Reynolds, 1995). Like p120, members of this family contain a series of 42 amino acid motifs called Armadillo (ARM) repeats. p120 has a core of ten ARM repeats that form the ARM domain. Although the crystal structure of p120 ARM domain has not

been elucidated, the crystal structure for the β-catenin ARM domain has been solved (Huber et al., 1997; Huber and Weis, 2001). The ARM repeats form a rigid super helical structure with a positively charged groove, which forms the binding pocket for the relatively unstructured cytoplasmic tail of E-cadherin and for Tcf. Based on sequence similarities, it is likely that the ARM domain of p120 will fold and function in a similar manner to that of β-catenin and form the binding pocket for many of p120's binding partners. Consistent with this, the ARM domain of p120 is required for its ability to bind E-cadherin (Reynolds et al., 1994), Kaiso (Daniel and Reynolds, 1999), and tubulin (Roczniak-Ferguson and Reynolds, 2003).

p120 and Cadherin-Mediated Cell-Cell Adhesion

Cadherins form a large superfamily of cell-cell adhesion molecules (reviewed in (Angst et al., 2001; Nollet et al., 2000; Takeichi, 1995; Yap, 1998)). Type I and Type II cadherins (cadherins hereafter) are single-pass transmembrane proteins that mediate Ca^{2+}-dependent cell-cell adhesion. Through their extracellular domains, they bind other cadherin molecules in a homophilic manner, both in *cis* and *trans* orientations. They also bind to β-catenin through the catenin-binding domain of their cytoplasmic tails (Nagafuchi and Takeichi, 1989; Ozawa et al., 1990; Stappert and Kemler, 1994). β-catenin then connects β-catenin to the actin cytoskeleton, thereby linking cadherins and the actin cytoskeleton (figure 2) (Aberle et al., 1994; Hulsken et al., 1994). Many other proteins have been shown to associate with the cadherin adhesion complex, including receptor tyrosine kinases (Qian et al., 2004), non-receptor kinases (Kim and Wong, 1995; Piedra et al., 2003; Xu et al., 2004; Zondag et al., 2000), phosphatases (Brady-Kalnay et

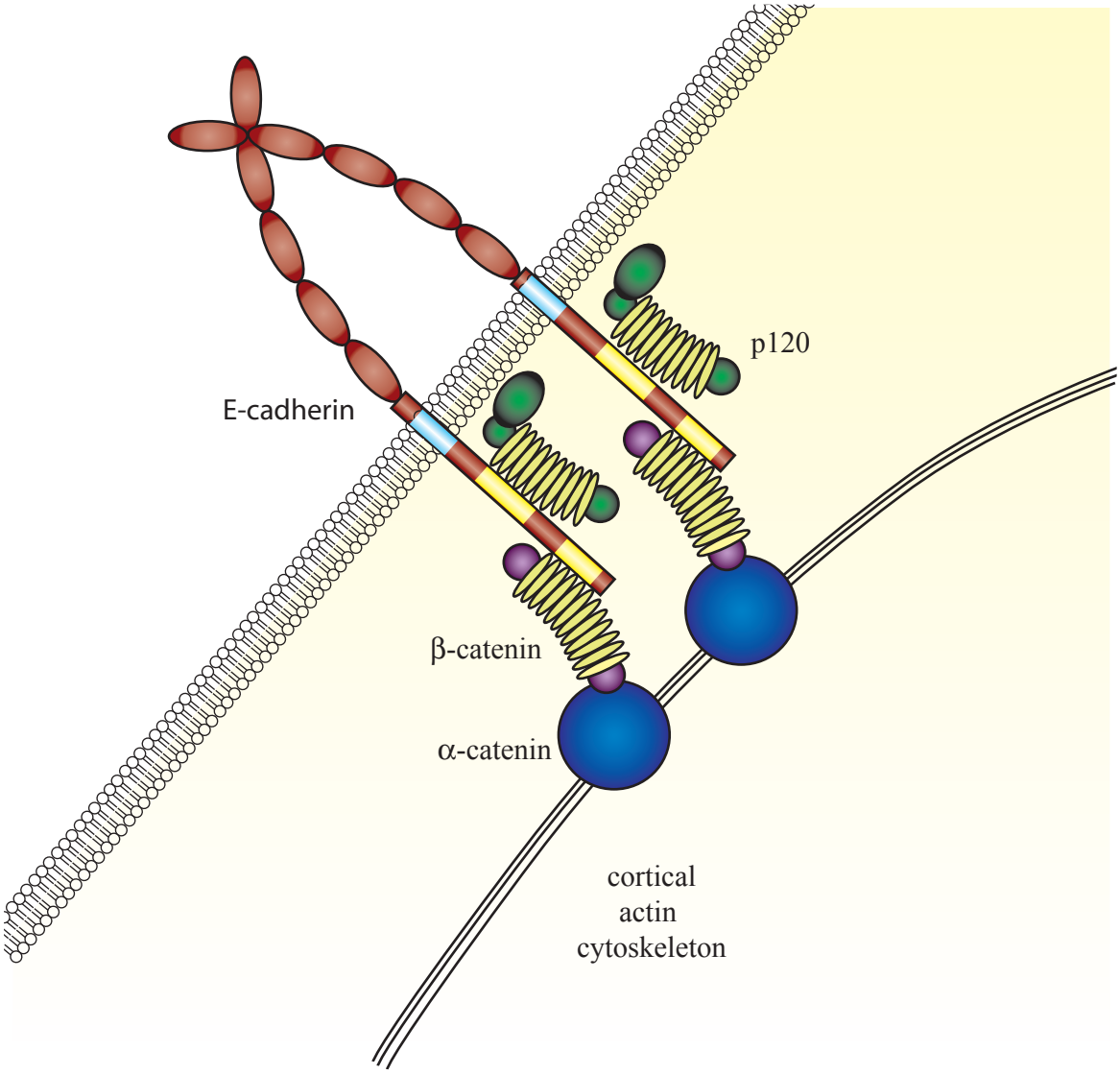


Figure 2. The E-cadherin Complex.

E-cadherin is a transmembrane cell-cell adhesion molecule that is regulated by its cytoplasmic binding partners, the catenins. p120 catenin binds to the juxtamembrane domain of E-cadherin, whereas β-catenin binds C-terminal catenin binding domain. α-catenin binds to β-catenin and links the cortical actin cytoskeleton to the cadherin complex. In a Ca^{2+}-dependent fashion, E-cadherin molecules dimerize in cis interactions with E-cadherin molecules on the same cells and in trans interactions with cadherin molecules on adjacent cells.

al., 1995; Keilhack et al., 2000). However, p120 is considered the final core member of the cadherin complex and binds the highly conserved juxtamembrane domain of type I and type II cadherins (Reynolds et al., 1992; Shibamoto et al., 1995; Staddon et al., 1995). Although p120 is not reported to connect to the actin cytoskeleton, it does regulate RhoA, Rac, and Cdc42, mediators of actin cytoskeletal dynamics(Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). Thus, cadherin complexes physically and functionally link the actin cytoskeleton of one cell to another.

Cadherin complexes form adherens junctions, which are found at the baso-lateral surface of polarized cells (Gumbiner and Simons, 1986). These junctions are generated when cadherin molecules at the cell surface link to the actin cytoskeleton through β-catenin and α-catenin, cluster together, and bind cadherin molecules on adjacent cells (reviewed in (Anastasiadis and Reynolds, 2000). However, even in apparently stable adherens junctions, these complexes are in a constant state of flux, as cadherin molecules transit from the cell surface to endocytic and exocytic vesicles (Bryant and Stow, 2004). This is due in part to the unusually short half-life of cadherin-cadherin interactions (Yap et al., 1997b; Yap et al., 1998). Thus the strength of the adherens junction is likely a result of the avidity of many cadherin complexes in the junction, rather than the affinity of individual cadherin molecules for one another.

Cadherins maintain the integrity of quiescent tissues (Takeichi, 1991) and are essential in embryogenesis (Kintner, 1992; Larue et al., 1994). Furthermore, loss of E-cadherin function is a transitional event leading to metastasis (Perl et al., 1998) (reviewed by (Nollet et al., 1999)). However, p120's role in regulating cadherin function is poorly

understood, and there is conflicting evidence suggesting p120 has either positive or negative regulatory roles in cadherin's adhesive functions.

In support of a positive role for p120 in regulating adhesion, deleting the juxtamembrane domain of C-cadherin, which is the E-cadherin homologue in Xenopus, decreases its adhesive strength (Yap et al., 1998). This suggests that the juxtamembrane domain is responsible for the time-dependent strengthening of adhesion associated with lateral clustering of cadherin molecules. More specifically, expressing an E-cadherin mutant that contains a three amino acid mutation of the juxtamembrane doamain and abrogates p120 binding, prevents epithelial colony compaction (Thoreson et al., 2000). Although cells expressing this E-cadherin mutant are able to adhere to one another, the adherens junctions are much weaker than those in cells expressing wild-type E-cadherin. Thus, p120 appears to function in strengthening cell-cell adhesion by potentially affecting lateral clustering of cadherin molecules.

Colo 205 cells do not typically form cell-cell contacts, despite having an intact cadherin complex. Treatment of Colo 205 cells with the kinase inhibitor staurosporine restores cell-cell adhesion, concurrent with a change in the phosphorylation of p120. Expressing an N-terminal deletion mutant of p120 that lacks the phospho-regulatory domain also allows aggregation of Colo 205 cells, whereas expressing full-length p120 does not restore cell-cell adhesion. (Aono et al., 1999). Similar results are observed in L-cells that either express a juxtamembrane mutant of E-cadherin or that co-express wild-type E-cadherin and the N-terminal deletion mutant of p120 (Ohkubo and Ozawa, 1999; Ozawa and Ohkubo, 2001). Collectively, these data indicate that p120 can negatively regulate cadherin function by signals received through its phospho-regulatory domain.

Much of the data demonstrating a role for p120 in cadherin function relies on juxtamembrane deletions of various cadherin molecules such as E-cadherin. Interpretation of these data are limited, however, as the juxtamembrane domain of E-cadherin also binds Presenilin-1 (Baki et al., 2001) and the E3 ubiquitin ligase Hakai (Fujita et al., 2002), making these data inconclusive. To directly establish a role for p120 in the cadherin complex, p120 negative cells were needed. The SW48 colonic adenocarcinoma cell line is the first purported to be p120 negative (Ireton et al., 2002a). Interestingly, these cells display a cell-cell adhesion defect, growing in linear arrays rather than the tightly clustered colonies most often observed with epithelial cell lines. We hypothesized that restoring p120 would also restore cell-cell adhesion. However, these cells do indeed express p120. Each allele in these cells is mutated, giving rise to a premature stop codon in one allele and a splicing mutation leading to the inclusion of several C-terminal introns in the other. The inaccuracy of the original claim suggesting these cells contained no p120 is due to the use of a C-terminal antibody whose epitope is lost by the mutations found on both alleles. Using several N-terminal antibodies reveals extremely low levels of p120 as compared to the colonic adenocarcinoma line HCT116. Therefore, in the SW48 cells, the adhesive defect may still be due to p120 loss.

Retroviral expression of wild type p120 in the SW48 cell line restores colony morphology of the infected cells to that of normal epithelia *in vitro*. Importantly, in SW48 cells expressing wild type p120, E-cadherin staining appears stronger and cells circumferentially connect with their neighbors, instead of only making contacts with two to three adjacent cells as in parental SW48 cells. Parental SW48 cells express lower levels of E-cadherin as compared to the control line HCT116, however upon p120

overexpression, E-cadherin levels rise dramatically. Furthermore, the ability to restore E-cadherin levels requires direct p120-E-cadherin interactions. This effect on E-cadherin is post-transcriptional, in that mRNA levels of E-cadherin are the same regardless of p120 levels. Finally, isoform 4, which lacks the phospho-regulatory domain, is better able to rescue the adhesion defect than either isoform 1 or 3. These data are the first to demonstrate that p120 is required for E-cadherin function and show that p120 also possesses a negative regulatory domain that may allow precise control of cadherin function. However, these results were generated in a single cell line, and these proposed roles for p120 can neither be generalized to all cell types nor to any cadherin besides E-cadherin.

Signaling and the Cadherin Complex

The cadherin complex is dynamically regulated to enable junction assembly and disassembly necessary for development and wound healing, however aberrant regulation of the cadherin complex can also force junction disassembly during tumor progression, leading to metastasis (Nollet et al., 1999). Thus, it is necessary to understand the signaling pathways associated with the cadherin complex. These pathways include: receptor tyrosine kinase activation (Qian et al., 2004), contact inhibition of cell growth (St Croix et al., 1998), regulation of the Rho family of small GTPases (Braga et al., 1997; Kim et al., 2000; Kodama et al., 1999; Takaishi et al., 1997), Wnt signaling (Huelsken et al., 2000; Kelly et al., 1995; Willert and Nusse, 1998), and Kaiso-mediated transcriptional regulation (Daniel and Reynolds, 1999; Kim et al., 2004; Prokhortchouk et

al., 2001). Although these pathways have been described here as discrete events, they are likely interrelated.

Phosphorylation of the E-cadherin complex, mediated by such effectors as Ca^{2+} influx, growth factors, and oncoproteins, correlates with changes in cell-cell adhesion. In fact, the growth factor receptors EGFR, Neu, and IGFR physically interact with E-cadherin and their interaction is density independent (Qian et al. 2004). Furthermore, E-cadherin-mediated adhesion inhibits ligand-induced activation of these receptors. Consistent with this, EGF-dependent phosphorylation of p120 increases with cell density (Mariner et.al). Because both growth factor receptors and cadherin complexes are located baso-laterally, it is likely that cell-cell contact limits the access of ligands to receptors and that receptors are primarily available to ligands during wounding, epithelial-mesenchymal transitions, etc.

Increasing evidence places the small GTPases (Rho A, Rac, and Cdc42) and cadherins in a common signaling pathway, where cadherins affect GTPase activity and *vice versa* (Braga, 2000; Braga, 1999; Braga et al., 1999; Braga et al., 1997; Jou and Nelson, 1998; Takaishi et al., 1997). Recently, our lab and others have suggested that this pathway may involve p120, as p120 was shown to inhibit Rho A and activate Rac and Cdc42 (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). These studies suggest that a non-cadherin-bound pool of p120 increases cell migration through regulation of these GTPases (Grosheva et al., 2001). Interestingly, dorsal injection of p120 into *Xenopus* embryos produces morphological effects on embryogenesis; whereas ventral injections cause no developmental defects (Geis et al., 1998; Paulson et al., 1999). As dorsal cells are reported to be more motile and more susceptible to changes in

motility(Keller and Danilchik, 1988), it is thought that p120 affects gastrulation by influencing cellular migration, which is consistent with a role for p120 in regulating the small GTPases. These studies raise the possibility that increased metastasis associated with E-cadherin loss is due not only to the loss of cadherin-based cell adhesion, but also to the ability of cytoplasmic p120 to promote cell motility.

In addition to its role in the cadherin adhesion complex, β-catenin is also a potent mediator of Wnt-induced transcriptional repression and activation (Huelsken et al., 2000; Kelly et al., 1995; Willert and Nusse, 1998). Typically, most β-catenin is bound to a cadherin, and remaining free β-catenin is phosphorylated and targeted for degradation by the APC/GSK3β complex (Aberle et al., 1997; Orford et al., 1997; Rubinfeld et al., 1996; Yost et al., 1996). However, Wnt-mediated signaling inhibits GSK3β, preventing its phosphorylation of β-catenin. Thus, cytoplasmic levels of β-catenin rise and β-catenin translocates to the nucleus. Interestingly, artificially induced EMT results in complete loss of E-cadherin and a concomitant 70% loss of β-catenin (Eger et al., 2000). Despite this dramatic reduction in total β-catenin levels, nuclear levels of β-catenin and the transactivation capacity of β-catenin are increased, leading to an increase in proliferation. This suggests that loss of E-cadherin during tumor progression may be accompanied by an increase in β-catenin-dependent transactivation and proliferation.

Like β-catenin, p120 also translocates to the nucleus, where it may functionally interact with Kaiso, a novel zinc finger transcriptional repressor that was originally discovered as a p120 binding partner *(Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999)*. Nuclear localization of p120 has been shown to alleviate Kaiso-specific transcriptional repression (Kelly et al., 2004), and in *Xenopus,* p120 and Kaiso

interactions modulate non-canonical Wnt signals (Kim et al., 2004). Little else is known regarding Kaiso and the role of p120 in the nucleus. However, p120 may parallel β-catenin with dual roles in cell-cell adhesion and transcriptional regulation.

Based on the above data, it is obvious that the cadherin complex is more than a static anchor, holding one cell to another, and that any work addressing the adhesive functions of the cadherin complex must also consider the roles that signaling may play in observed phenotypes. Furthermore, loss of p120 may profoundly affect more than just cell-cell adhesion and could disrupt such signaling pathways as growth factor-mediated signaling, Wnt signaling, Non-canonical Wnt signaling, and Rho GTPase function.

p120 and the Cadherin Complex in Development

To understand the role of the cadherin complex in development and organogenesis in vivo, murine E-cadherin, β-catenin, α-catenin, and p120 have been completely eliminated or, as in the case of β-catenin, mutated to prevent association with the complex (Huelsken et al., 2000; Larue et al., 1994; Torres et al., 1997). E-cadherin is found in cells at the earliest stages of development (Hyafil et al., 1980; Vestweber et al., 1987). Prior to the establishment of apico-basal polarity at the 8-cell stage of murine development, E-cadherin localizes uniformly over the entire cell surface. Upon apico-basal polarization, E-cadherin relocalizes to the sites of cell-cell contact and is probably required for embryonic compaction, which occurs at approximately the 16-cell stage. However, loss of E-cadherin and β-catenin function do not affect apico-basal polarization or embryonic compaction (Larue et al., 1994; Torres et al., 1997). Instead, animals die prior to implantation in the uterine wall, fail to form the first differentiated embryonic

epithelium, and are unable to form and maintain a blastocyst cavity. Failure to affect polarization and compaction is likely due to the persistence of maternal E-cadherin throughout these stages; nevertheless, these data show the absolute requirement of E-cadherin and β-catenin in embryonic development.

β-catenin knockouts die at e8.5 and show defects in anterior-posterior axis development (Huelsken et al., 2000). However, deleting β-catenin has no effect on either E-cadherin staining patterns or on the formation of adherens junctions. Plakoglobin is a close family member of β-catenin that typically binds to desmosomal cadherins; however, it can also bind to the catenin-binding domain of E-cadherin. Interestingly, in the β-catenin knockout animals, plakoglobin levels increase, and plakoglobin localizes to the adherens junctions. Thus, the anterior-posterior defects are attributed to aberrant Wnt signaling, as cadherin function is apparently unaffected in these animals.

Finally, p120 knockouts die between e9.5 and e12.5 with a thin and discontinuous notochord (personal communication from Walter Birchmeier, Berlin). Although immunolocalization of cadherin complex members was not examined, ultrastructural observations reveal fewer adherens junctions between cells of the notochord. It is unclear as to why p120 knockouts die so much later than E-cadherin and β-catenin mutant animals. However, p120 family members, ARVCF and p0071, may compensate for p120 loss in the cadherin complex up until e9.5 (Ireton et al., 2002b). These studies show that p120, like E-cadherin, is an essential gene and is required for normal development and embryogenesis.

The Importance of p120 and the Cadherin Complex in Tumor Progression

Arguably, metastatic transformation requires the loss or deregulation of cell-cell adhesion (Cavallaro and Christofori, 2004), and indeed, E-cadherin expression is lost in nearly half of the metastatic tumors. In tumors, functional E-cadherin loss occurs primarily through deletion of the gene, through transcription-blocking hypermethylation, and through overexpression of transcriptional repressors such as Snail. However, inactivating mutations in sporadic and familial gastric cancer and sporadic lobular carcinoma of the breast also prevent cell-cell adhesion (Nollet et al., 1999). Finally, E-cadherin expression induces mesenchymal-to-epithelial reversions in carcinoma cell lines (Auersperg et al., 1999; Chen et al., 1997) and can block progression of known cancer models at stages prior to metastasis. Thus E-cadherin is a tumor/metastasis suppressor, whose loss is considered a key event in the progression towards metastasis.

The loss of E-cadherin appears to be the most frequent manner a tumor cell employs to disrupt cell-cell adhesion (Nollet et al., 1999). However, as proper cell-cell adhesion requires a link to the actin cytoskeleton through α- and β-catenin, any mutation that disrupts this connection could promote metastasis. Consistent with this hypothesis, there are mutations in β-catenin in signet cell carcinoma that prevent β-catenin binding to α-catenin (Kawanishi et al., 1995). Likewise, inactivating mutations and deletions of α-catenin have been reported in a wide number of cancer cell lines, and re-expression of α-catenin restored cell-cell adhesion in many of these lines (Nollet et al., 1999). Collectively, these data indicate the importance of the cadherin adhesion complex as a whole in tumor progression.

While many data suggest that p120 plays a role in tumor progression, p120 is not yet considered a bona fide tumor modifier. For instance, E-cadherin recruits 90% of the p120 protein pool to the membrane, yet p120 stably localizes to the cytoplasm when E-cadherin is lost (Thoreson et al., 2000). Additionally, p120 can increase cell motility through regulation of RhoA, Rac, and Cdc42--GTPases and oncogenes that are mediators of the actin cytoskeleton (Grosheva et al., 2001; Noren et al., 2001). Consistent with these observations, E-cadherin loss often correlates with cytoplasmic localization of p120 in tumors, where it may promote metastasis through regulation of the Rho GTPases (Thoreson and Reynolds, 2002). However, recent studies in the SW48 carcinoma cell line show that p120 loss is responsible for a severe reduction in E-cadherin levels (Ireton et al., 2002a), and there are reports of concurrent p120 and E-cadherin loss in tumors of the bladder, stomach, breast, prostate, lung, and pancreas (Thoreson and Reynolds, 2002). Therefore, in certain cases p120 loss may actually cause E-cadherin loss in tumors. While these data clearly suggest a role for p120 in tumor progression, either through the activation of Rho GTPases or through the destabilization of E-cadherin, they do not conclusively implicate p120 in the disease process.

Hypothesis

p120 re-expression in SW48 cells dramatically increases E-cadherin levels and restores epithelial morphology, and there is a tight correlation between p120 loss and E-cadherin loss in tumors. Therefore, I hypothesize that p120 loss will lead to E-cadherin loss, and when this occurs *in vivo*, it may further tumor progression towards a more malignant phenotype. To test this hypothesis, I have generated p120-specific siRNA

constructs to examine p120 loss *in vitro*, and I have generated a conditional knockout of murine p120 to study p120 loss *in vivo*.

CHAPTER II

MATERIALS AND METHODS

Cell Culture, Infections, and Transfections

HUAEC's (Cambrex, CC-2535) were thawed at passage one. They were grown in EBM Basal Medium (Cambrex, CC-3121) supplemented with EGM Singlequots Supplements and Growth Factors (Cambrex, CC-4133). Just prior to use, HUAEC culture dishes were treated with 0.2% Gelatin (Sigma, G1393) in PBS for 20 min. at 37° C. Culture conditions for Phoenix cells (Ireton et al., 2002a) have been described, and all other cell lines were cultured as described (Anastasiadis et al., 2000). For siRNA expression, cells were infected with pRS and selected with 3 to 5 □g/ml Puromycin. As indicated, some cells were infected again with LZRS-mp120-Neomycin and selected with 600 □g/ml Neomycin. pRS and LZRS retroviruses were produced in the Phoenix cell packaging line as described (Ireton et al., 2002a). Clonal A431 cell lines were subcloned by limiting dilution. p120 expression was assessed by immunofluorescence and Western blotting. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

Immunofluorescence and FAC sorting

Cells were plated sparsely on glass coverslips and incubated for 2 days before immunofluorescent labeling. Cells were washed once with PBS, then fixed in 3% Para formaldehyde for 30 min. Fixed cells were washed with PBS/glycine twice and

permeabilized in 0.2% Triton-X/PBS for 5 min. Cells were again washed in PBS/glycine and blocked in 3% milk/PBS before staining. Primary antibodies mAb pp120 (Transduction Labs), anti-α-catenin (C-2206, Sigma), anti-β-catenin (C-2081, Sigma), anti-E-cadherin C-20820 (Transduction Labs), and HECD-1 (kind gift from Masatoshi Takeichi, Kobe, Japan) were used as described previously (Ireton et al., 2002a). Other primary antibodies were used as follows: Anti-tubulin (Sigma, DM1a) 1:1000, anti-vinculin (Sigma, hvin-1) 1:400, anti-myc (mAb 9E10) 1μg/ml and SHE78-7 anti E-cadherin (Zymed) 1μg/ml. Secondary antibody concentrations were as described (Ireton et al., 2002a). Cells were mounted in Prolong Antifade (Molecular Probes) according to manufacturer's instructions.

To isolate pools of cells expressing different levels of E-cadherin, a p120 siRNA infected A431 cell population was sorted by FACS (Fluorescent Activated Cell Sorting) as follows. Cells were dissociated with Gibco cell dissociation buffer (enzyme free, PBS based) at 37°C for 45 minutes. Single cell suspensions were enhanced by repeated pipetting, washed in PBS containing 1% serum, and then labeled with E-cadherin mAb HECD1 (10 μg for 5 x 10^6 cells in 1 ml), followed by washing and then additional labeling with the secondary antibody Alexa-488 conjugated goat anti mouse IgG (1/1000 dilution in 1 ml)(Molecular Probes). After washing, cells were labeled with 7AAD (Molecular Probes) to discriminate dead cells, and subjected to FACS using a FACSTAR plus cell sorter (Becton Dickinson). All procedures were carried out at 4°C to prevent E-cadherin endocytosis. Four gates were set based on preliminary experiments designed to separate cells into four categories of cells expressing high to low levels of E-cadherin.

The resulting pools were expanded and then analyzed by Western blotting for p120 and E-cadherin levels.

Pulse Chase, Biotinylation, and Cell Surface Trafficking

Pulse chase experiments were carried out exactly as described (Ireton et al., 2002a). Biotinylation and the rate of cell surface trafficking were performed exactly as described (Bonifacino and Jackson, 2003). Briefly, cells were plated at 5 x 10^5 cells per 60 mm dish for 36 hours prior to pulse chase. Cells were labeled with ^{35}S for 15 min prior to chase. At the end of the chase, cell surface proteins were labeled with 1mg/ml EZ-Link sulfo-NHS-SS-biotin (Pierce) at 4°C for 30 min. E-cadherin was immunoprecipitated from NP-40 cell lysates and surface cadherin was detected by dividing the E-cadherin immunoprecipitations in half, eluting E-cadherin from the beads with 0.5% SDS, reconstituting elutions in 50 mM Tris-HCl (pH 7.5), and pulling down biotinylated E-cadherin with 10 l per sample of packed streptavidin coated agarose beads (Sigma) for 1 hr at 4°C. Samples were washed three times with 50 mM Tris-HCl (pH 7.5), protein eluted with 2x LSB, and analyzed by SDS-PAGE and autoradiography as described (Ireton et al., 2002a). Quantification was performed by densitometry using Image Gage software (Fujifilm Inc.). Arbitrary densitometry units were plotted with Prism Graph (Graphpad Software Inc.), and adjusted for background. Biotinylation of total surface cadherin was performed as described above but without the pulse chase labeling.

Constructs

LZRS-mp120-Neo has been described in detail (Ireton et al., 2002a). The pRS vector was a gift from Reuven Agami (Netherlands, Amsterdam). pRS human p120 siRNA and pRS murine p120 siRNA were generated according to Brummelkamp et al. (Brummelkamp et al., 2002). Briefly, a 64 bp linker was inserted into pRS using the BamHI and HindIII sites. Oligos for the linker contained p120-specific sense and corresponding antisense sequences, flanking a 6 base hairpin and were PAGE purified by IDT. pEGFP-C1 β-catenin (Lu et al., 1999) was a gift from Qun Lu (Greenville, NC). pEGFP-C2 ARVCF C11 (Waibler et al., 2001) was a gift from Anna Starzinski-Powitz (Frankfurt, Germany). pEGFP-C1 (Clontech) was used as a negative control in transfection experiments.

Western Blotting

Western blotting procedures were conducted as described by Mariner et al. (Mariner et al., 2001). Briefly, cells were grown to confluence and lysed with either NP-40 or RIPA buffer. Protein concentrations in lysates were obtained by BCA assay (Pierce Chemical Co.) according to manufacturer's instructions. The following primary antibodies were used as follows: mAb pp120 (Transduction labs, 0.1 μg/ml), anti-E-cadherin mAb's C-20820 (Transduction Labs, 1/2,500) and HECD-1 (kind gift from Masatoshi Takeichi, Kobe, Japan, 0.1 μg/ml), anti-β-catenin pAb (Sigma, C2206, 1/5,000), and anti-α-catenin pAb (Sigma C-2081, 1/5,000). Secondary antibodies were peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, 1/10,000) and

mouse anti-rabbit IgG (Jackson Immunoresearch, 1/10,000). Anti-tubulin (Sigma, DM1a) and anti-vinculin (Sigma, hvin-1) were used at 1:1000 and 1:400 respectively.

Inhibitors

Cells were plated at 5 x 10^5 cells per 60 mm dish for 36 hours prior to treatment with inhibitors. Inhibitors were added to standard growth media at the following concentrations: 33 nM PS341(Millennium Pharmaceuticals), 3.3 μM lactacystin (Calbiochem), 33 μM chloroquine (Sigma), 5 mM ammonium chloride (Sigma), and 10 nM IETD-CHO (Calbiochem). Cells were treated with inhibitors for 24 hr prior to lysis in NP-40 buffer and analyzed by Western blotting with E-cadherin mAb HECD-1.

Generation of Targeting Construct

Genomic DNA for murine p120 was obtained by screening the RPCI-22 (129S6/SvEvTac) mouse BAC library with a cDNA probe generated against full-length murine p120 isoform 1A. Intronic probes were generated from p120-containing BAC clones and used to screen a shotgun library of HindIII-digested BAC fragments. Sequences obtained from plasmids that stained positive for p120 fragments were used to create a sequence map spanning intron 2 to exon 15 (see Chapter 4, figure 1A).

To generate the Neo-TK selection cassette, TK was removed from the pPNT vector (Tybulewicz et al., 1991) by EcoR1-HindIII digestion, then inserted immediately downstream of the Neo cassette of pM30 (Meyers et al., 1998) after blunt-ending both vector and insert. The targeting construct was pieced together through numerous steps, but briefly it begins at the second PshA1 site upstream of exon 3 and ends with the PmlI

site following exon 15. The selection cassette containing Neo, TK, and the 5' LoxP site was inserted into the first PshA1 site upstream of exon 3, and the 3' LoxP site was inserted into an Asc1 site generated between two BbvC1 sites in intron 8.

Generation of floxed p120 mice

206 μg targeting construct illustrated in figure 1 was linearized with NotI and electroporated into 35 X $10^6$129SvEvTac ES cells. Stable integrants were selected with 0.2 mg/mL G418. Twenty-five homologous integrants were identified by screening 384 KpnI-digested G418-resistant clones with a 681 bp PCR-generated probe (5'-CTT GTG CTG TTA TTT GGT GAC TGG-3' to 5'-CGT CTG TAA TCC CTC TGC TTG TGA G-3') illustrated in figure 1A. Of the 25 homologous integrants, 6 were chosen for FlpE-mediated recombination to remove the selection cassette (100 μg p06 FlpE 6 into 2.0 X 10^6 cells); these were negatively selected with 0.2 μM gancyclovir. Resistant clones were screened by PCR, and three recombinants were isolated from nearly 1000 clones screened. All three clones were from the same parental G418-resistant ES cell clone. Using standard procedures, one ES-cell clone was used to successfully generate a founder line.

Genotyping was accomplished by isolating DNA with Sigma's RedextractNAmp tissue PCR kit (#XNAT), then screening for desired genes by PCR. The primers for MMTV-Cre (Wagner et al., 1997) have been described previously. The 3' LoxP site for p120 was identified with 5'-TTT TAG AGC CTC CCA CAT ACA AGC-3' and 5'-TCA GCA CCC ACA CAA AGG TTG-3' which identified a 322 bp wild type allele and a 365 bp floxed allele, while the 5' LoxP site was identified with 5'-TTG AAC TCA GGA

CCG TCA GAG GAG-3' and 5'-AAA GCA AGC CAC CAC CAA CC-3' to identify a 450 bp wild type and a 564 bp floxed allele. The 5' LoxP primer set was used routinely for genotyping.

Immunohistochemistry

Tissues were fixed in formalin overnight at 4 °C. Using standard techniques, 5 m paraffin-embedded sections were prepared for H&E staining, PAS staining, and Immunohistochemistry (IHC). For IHC we used Sodium Citrate antigen retrieval for all antibodies except Occludin and BrdU. Antigen retrieval for occludin was achieved by incubating sections in 1mg/ml Protease (Sigma P5147) for 10 minutes at 37 degrees. BrdU staining required 2N HCl at 37 °C for 20 minutes, 1% boric acid/1% borate for 1 minute at 37 °C, and 5 g/ml trypsin for 3 minutes at 37 °C. For fluorescent IHC, non-specific epitopes were masked with 10mM Tris-HCl pH 7.4, 0.1M $MgCl_2$, 0.5% Tween 20, 1% BSA, and 5% normal goat serum. We then diluted antibodies in this blocking solution and added them to samples in a humidified chamber. Samples were incubated with primary and secondary antibodies overnight at 4 °C and 2 hours at room temperature, respectively. Sections were mounted with Prolong Antifade Mounting Medium (Molecular Probes #P7481). Finally, we visualized staining on a Zeiss Axioplan 2 microscope and collected images using Open Lab software (Improvision) and a Hamamatsu Orca-ER digital camera (#CA742-95); all images were pseudo-colored. Non-fluorescent IHC used standard techniques.

We stained sections with the following primary antibodies: Rabbit anti-p120 (F1aSH, 1:400, (Thoreson et al., 2000)); anti-p120 Catenin/pp120 (#610133, 1:400) and

anti-E-cadherin (#610181, 1:500) from BD Transduction Laboratories; anti-□-catenin (#C2206, 1:800), and anti-□-catenin (#C2066, 1:800) from Sigma; Na^+, K^+-ATPase (Upstate biotechnology #05-369, 1:1000); Desmoglein 1 (Santa Cruz, #sc-20114; 1:200); Occludin (Zymed #71-1500, 1:50); BrdU (Accurate Chemical & Scientific Corp. #OBT0030, 1:400); anti mouse neutrophil (Serotec #MCA771GA, 1:150); and Crumbs 3 (kind gift from Ben Margolis, Ann Arbor, MI, 1:400,(Makarova et al., 2003)). Apoptotic events were detected with the ApopTag Fluorescein In Situ Apoptosis Detection (Chemicon # S7110).

Secondary Antibodies, conjugated to the Alexa-fluor, were obtained from Molecular Probes and emitted light at 488 nm or 594 nm.

Electron Microscopy

Wild type and knockout 1dpp salivary tissues were removed and placed in room temperature 2.5% glutaraldehyde for at least 1 hour before storing overnight at 4°C. Specimens were then rinsed in cacodylate buffer, fixed in 1% osmium tetroxide, and rinsed again. Samples were dehydrated and transitioned into the Spur embedding resin through a one to one solution of propylene oxide and Spur. Once in Spur, samples were incubated overnight under vacuum before embedding in fresh Spur. Finally, embedded samples were hardened at 60° C overnight. 80 nm thin sections were viewed on a Phillips EM400 electron microscope, operated at 80 keV.

CHAPTER III

A CORE FUNCTION FOR P120-CATENIN IN CADHERIN TURNOVER

Authors note

This work was accomplished as an equal collaboration between Reneé Ireton and myself.

Introduction

p120-catenin (hereafter p120) is the prototypic and most abundant member of an Arm domain protein subfamily that includes ARVCF, δ-catenin, and p0071 (reviewed in (Anastasiadis and Reynolds, 2000). p120 was originally described as a substrate for Src- and Receptor-Tyrosine Kinases (Reynolds et al., 1992; Reynolds et al., 1989), and later identified as a catenin (Reynolds et al., 1994; Shibamoto et al., 1995), one of several cofactors that interact with the cadherin tail and modulate cadherin function (reviewed in Anastasiadis and Reynolds, 2000). The classical catenins, α- and β-catenin, bridge the cadherin cytoplasmic domain to the underlying actin cytoskeleton. p120 is required to stabilize E-cadherin in SW48 cells (Ireton et al., 2002a) and may also regulate cadherin – cytoskeletal connections indirectly through functional interactions with Rho GTPases (Anastasiadis et al., 2000; Grosheva et al., 2001; Magie et al., 2002; Noren et al., 2000) (reviewed in Anastasiadis and Reynolds, 2001), but the underlying mechanisms have not been established.

E-cadherin is the main cell-cell adhesion molecule in epithelial tissues and is regarded as a master organizer of the epithelial phenotype (Takeichi, 1995). Direct

mutation of the E-cadherin gene in gastric and lobular breast carcinomas indicates a classical tumor suppressor role in some tumors (Berx et al., 1995; Oda et al., 1994). In late stage carcinomas of all types, E-cadherin downregulation occurs frequently via epigenetic mechanisms (Comijn et al., 2001; Matsumura et al., 2001) and is closely correlated with the transition to metastasis (Birchmeier and Behrens, 1994; Frixen et al., 1991; Perl et al., 1998; Vleminckx et al., 1991). Together, these data establish E-cadherin as a tumor and/or metastasis suppressor, depending on the mechanism and timing of E-cadherin downregulation (reviewed in Nollet et al., 1999; reviewed in Yap, 1998).

In the event of E-cadherin downregulation, □□ and □-catenins are rapidly degraded (Nagafuchi et al., 1991) via an Adenomatous Polyposis Coli (APC)-dependant mechanism (Polakis, 2000) that ultimately targets □-catenin for destruction by the proteosome (reviewed in Kikuchi, 2000). In contrast, p120 is stable in the absence of cadherins and becomes stranded in the cytoplasm (Thoreson et al., 2000). We, and others, have postulated that cytoplasmic p120 actively drives the metastatic phenotype in cadherin-deficient cells through inappropriate activation/suppression of various Rho-GTPases such as Rac1 and RhoA (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001; Grosheva et al., 2001; Noren et al., 2000). These data suggest a metastasis promoter role for p120 when mislocalized through prior loss of E-cadherin.

Downregulation of p120-catenin occurs frequently in colon, prostate, breast, lung, and other carcinoma types (reviewed in Thoreson and Reynolds, 2002), but the consequences are unknown. Paradoxically, it is rare to see p120 downregulation in established tumor cell lines. The lone exception is the SW48 colon carcinoma cell line,

where genetic alterations result in extremely low levels of a mutated p120 that lacks the carboxy-terminus (Ireton et al., 2002a). Restoring normal levels of full-length p120 expression in these poorly organized cells stabilized E-cadherin and caused a striking rescue of epithelial morphology. Thus, in SW48 cells at least, p120 appears to be essential for E-cadherin stability and function (Ireton et al., 2002a). On the other hand, recent reports in *Drosophila* (Myster et al., 2003; Pacquelet et al., 2003) and *C. elegans* (Pettitt et al., 2003) indicate that p120 is not essential and its absence causes only minor defects that are not fully apparent unless complemented by weak alleles of E-cadherin or β-catenin.

Here, to clarify the role of p120 in mammalian cells, we have knocked-down p120 with siRNA in cells expressing Epithelial (E), Placental (P), Neuronal (N), and Vascular Endothelial (VE) cadherins. We report that each of these cadherins, as well as α- and β-catenins, were rapidly degraded in the absence of p120, resulting in loss of cell-cell adhesion. The effect was clearly dose dependent, indicating that p120 expression levels may directly determine cadherin levels. Degradation of p120-uncoupled cadherin occurred after its arrival at the surface, indicating that p120 regulates cadherin turnover at the level of internalization or recycling. p120 homologues ARVCF and δ-catenin could substitute for p120, so at least one family member is likely required to maintain adhesion. Thus, cadherin complexes are rapidly turned over and degraded in mammalian cells in the absence of direct interaction with p120 or a p120 family member. These observations establish a core function for p120 in the cadherin complex and have additional implications in support of a role for p120 in tumor suppression.

Results

p120-loss leads to loss of the cadherin complex

To directly address the general consequences of p120-deficiency, we stably expressed p120-specific siRNA using the pRetroSuper (pRS) retrovirus to knockdown p120 in mammalian cell lines (Fig. 1). Human and murine p120 siRNA's (h siRNA and m siRNA, respectively) were generated against homologous human and murine sequences that differ by 3 mismatches at the nucleotide level (Fig. 1a). Pilot experiments revealed that the human siRNA strongly knocked down p120 levels in human, but not murine cells, and vice versa. E-cadherin levels were also severely reduced by p120-knockdown in several different epithelial cell lines. These data indicate that the stabilizing effect of p120 is not limited to SW48 cells, but represents a mechanism that is likely common to all E-cadherin-expressing cells.

By intentionally targeting the above siRNA oligos to human and murine sequences that differed by several nucleotides, it was relatively straightforward to efficiently "knockdown" p120 with the human directed siRNA (pRS-h siRNA) and subsequently "knock-up" p120 by infection with pLZRS-mp120, a retrovirus containing the murine p120 cDNA (Fig. 1b). Restoring p120 levels by expressing murine p120 reversed the effects of the human siRNA and restored adhesion (Fig. 1b &c). It is worth noting that this method is generally applicable to any protein. If a homologous gene is not available, a knock-up construct can be generated by making silent mutations in the region targeted by the siRNA. The method is a simple *in vitro* equivalent of transgenic knockout and knock-in technology, and essentially solves the common dilemma

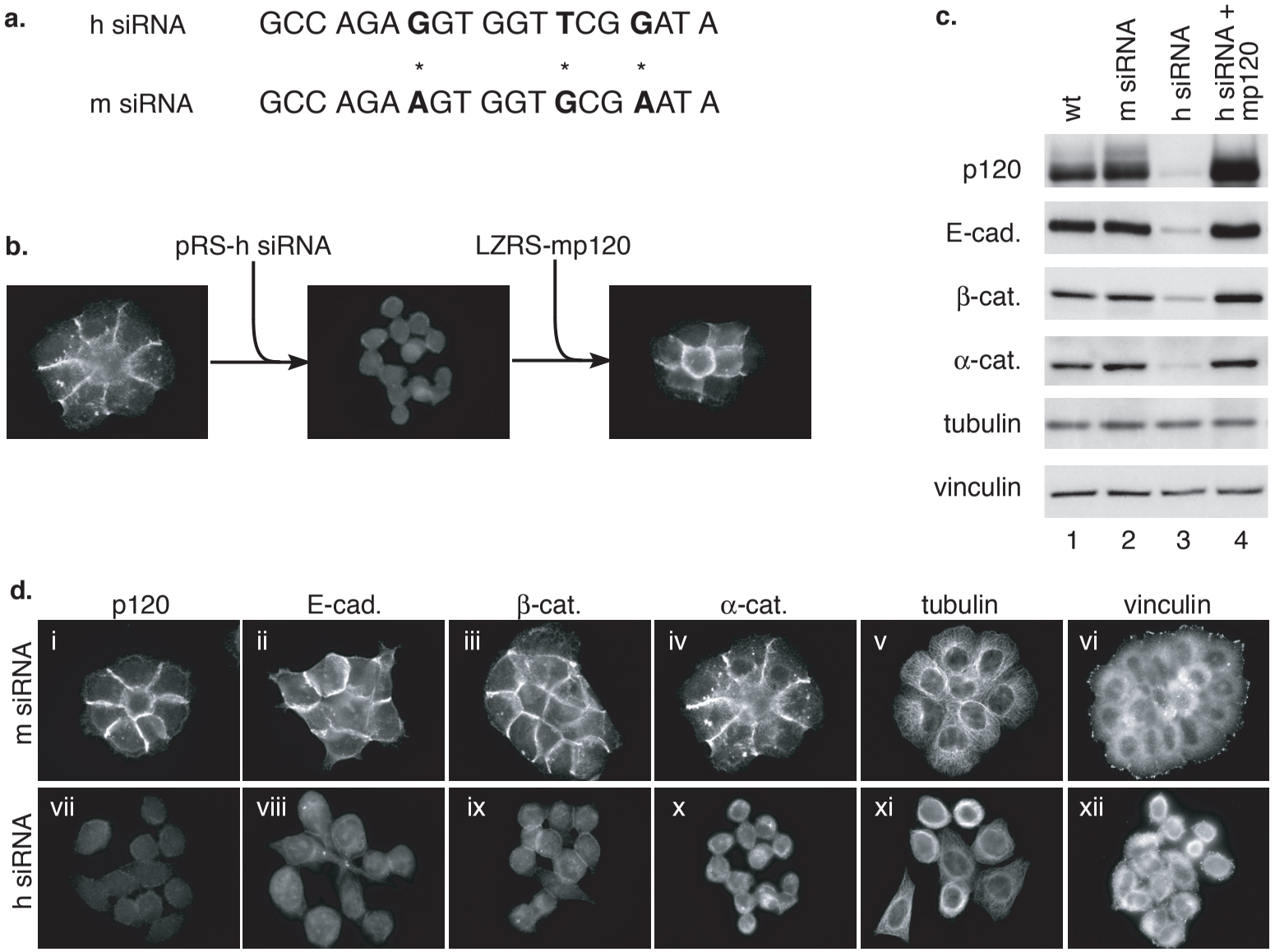

a.
h siRNA
GCC AGA GGT GGT TCG GAT A
m siRNA
GCC AGA AGT GGT GCG AAT A
b.
pRS-h siRNA
LZRS-mp120
c.
wt
m siRNA
h siRNA
h siRNA + mp120
p120
E-cad.
β-cat.
α-cat.
tubulin
vinculin
1
2
3
4
d.
p120
E-cad.
β-cat.
α-cat.
tubulin
vinculin
m siRNA
h siRNA
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii

Figure 1. p120 knockdown eliminates the E-cadherin complex and abolishes adhesion.
(a) Human and murine p120 siRNA's (h siRNA and m siRNA, respectively) were generated against homologous human and murine sequences that contain three mismatches at the nucleotide level (asterisks). (b) Schematic depicting a novel method for in vitro p120 "knockdown" and "knock-up". Human p120 was knocked down using the retroviral vector pRS to express human specific p120 siRNA and stable cell lines were selected. p120 was then re-expressed (knock-up) by infecting the knock down cell line with an LZRS retrovirus containing murine p120 cDNA. (c) wt-A431 cells (lane 1) were infected with virus carrying the control m siRNA (lane 2) or h siRNA (lane 3), and stable cell lines were isolated. p120 expression was restored (knock up) by infecting h-siRNA expressing cells with retrovirus containing murine p120 (lane 4). The indicated cadherin complex proteins were analyzed by Western blotting whole cell lysates. E-cadherin, β-catenin, and α-catenin levels were substantially reduced in p120 knockdown cells, and restoring p120 reversed the effect. (d) p120 (i, vii) E-cadherin (ii, viii) β–catenin (iii, ix) α–catenin (iv, x) tubulin (v, xi) and vinculin (vi, xii) were localized by immunofluorescence in stable A431 cell lines expressing the control m siRNA (i-vi) or h siRNA (vii-xii). Cells were plated sparsely to allow colonies to emerge from single cells. Note that p120 knockdown cells lack cadherin complexes and have lost cell-cell adhesion. The cadherin complex is selectively targeted since the levels of tubulin and vinculin are unaffected.

associated with expressing mutant proteins in cells that already contain high levels of an endogenous counterpart. This is the first example, to our knowledge, of this broadly applicable method.

To examine the effects of p120 knockdown in detail, we isolated stable clones of A431 cells expressing p120-specific siRNA and characterized them by Western blotting (Fig. 1c) and by immunofluorescence (Fig. 1d). p120 was nearly eliminated by human (Fig. 1c, lane 3), but not murine siRNA (lane 2), and p120-loss induced near complete loss of E-cadherin. Levels of □- and □-catenin were also severely reduced, as expected from the fact that these catenins are stabilized via interaction with cadherins (17). Thus, p120 loss essentially eliminated the entire cadherin complex. Levels of vinculin, which concentrate at focal adhesions in these cells, were unaffected, as were levels of tubulin.

Analysis of the p120-knockdown cells by immunofluorescence revealed near complete loss of junctional E-cadherin, loss of □- and □-catenins, and loss of cell-cell adhesion (Fig. 1d). It is noteworthy that other adhesion systems (e.g., desmosomes) cannot compensate for loss of the core components of the adherens junction. These observations reveal that p120 is essential for adhesion and suggest a core function for p120 in regulating cadherin turnover.

The requirement for p120 is common to other cadherins

To determine whether the consequence of p120 knockdown pertains only to E-cadherin, we repeated the experiments described in figure 1 on cells expressing E-, P-, VE-, and N-cadherins (Fig. 2). A431 (human cervical carcinoma), HUAEC (human umbilical aortic vascular endothelial), and C2C12 (murine myoblast) cells were selected

because they express E- and P-cadherin, VE-cadherin, and N-cadherin, respectively. Interestingly, the levels of each of these cadherins were substantially reduced by p120 knockdown (Fig. 2, lanes 3, 6, and 9). Note that because C2C12 cells are murine, the constructs are reversed relative to the human lines: murine siRNA is the knockdown construct and the human siRNA is the control. The knockdown levels in these experiments are not quite as striking as in the clonal cell lines represented in figure 1 because they are polyclonal cell lines and therefore represent the average siRNA expression and knockdown from multiple integration events. Nonetheless, these data indicate clearly that the mechanism of stabilization by p120 is common to a wide variety of cadherins, probably all cadherins that bind p120.

p120 levels directly determine cadherin levels

To more accurately quantify the relationship between p120 and cadherin expression, we infected A431 cells with the p120 siRNA virus and analyzed individual cell clones by co-immunofluorescence for p120 and E-cadherin (not shown). We also carried out the reverse experiment (knock-up), by introducing murine p120 into the human siRNA expressing A431 cells (Fig. 3). In all cases, there was a striking correlation between the levels of p120 and E-cadherin, which was also reflected by the extent of cell-cell adhesion. In the absence of p120 there was essentially no E-cadherin present (panels i and ii). By contrast, intermediate levels of p120 caused intermediate levels of E-cadherin and partial restoration of epithelial morphology (panels iii and iv). When murine p120 was expressed at higher than normal levels, E-cadherin levels were correspondingly elevated and exceeded the wild type levels observed in the parental cell

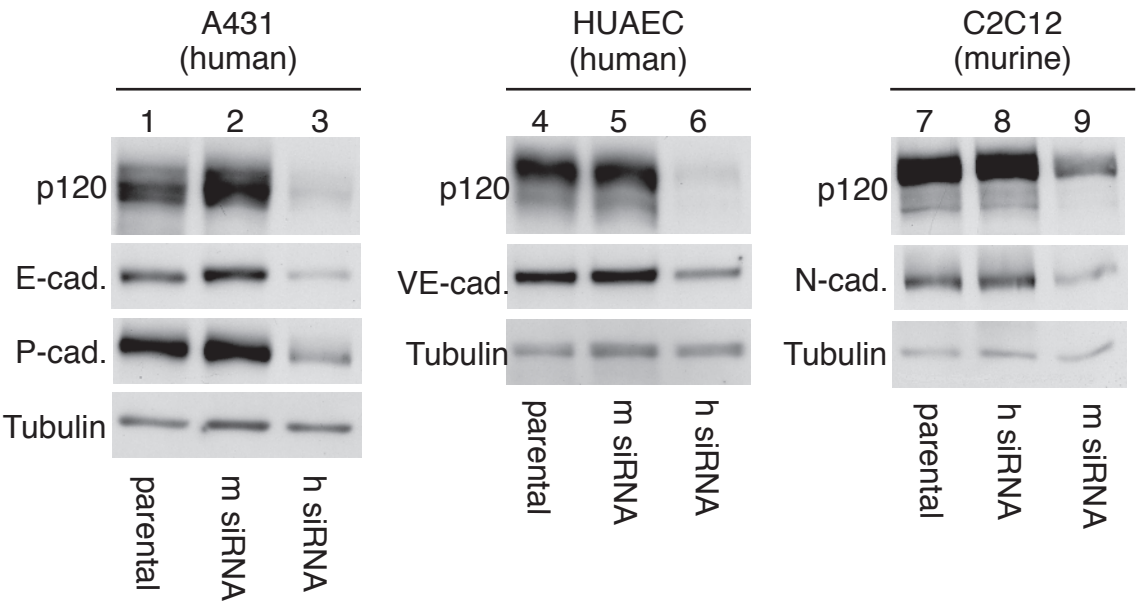


Figure 2. The p120-associated destruction mechanism is common to multiple cadherins. A431 (human cervical carcinoma), HUAEC (human umbilical aortic endothelial), and C2C12 (mouse myocyte) cells express E- and P-, VE- and N-cadherins respectively. Each cell line was infected with either human or murine specific p120 siRNA retrovirus to generate polyclonal knockdown cell lines, and levels of p120 or E-, P-, VE-, and N-cadherins were assayed by Western blotting of whole cell lysates. Tubulin levels were used as a loading control. p120 knockdown reduced expression of all of these cadherins, indicating that its function is common to most, if not all p120-associated cadherins. Note that the effects of the human and murine siRNA's used for knockdown and control in the human cell lines are reversed in the murine cell line C2C12.

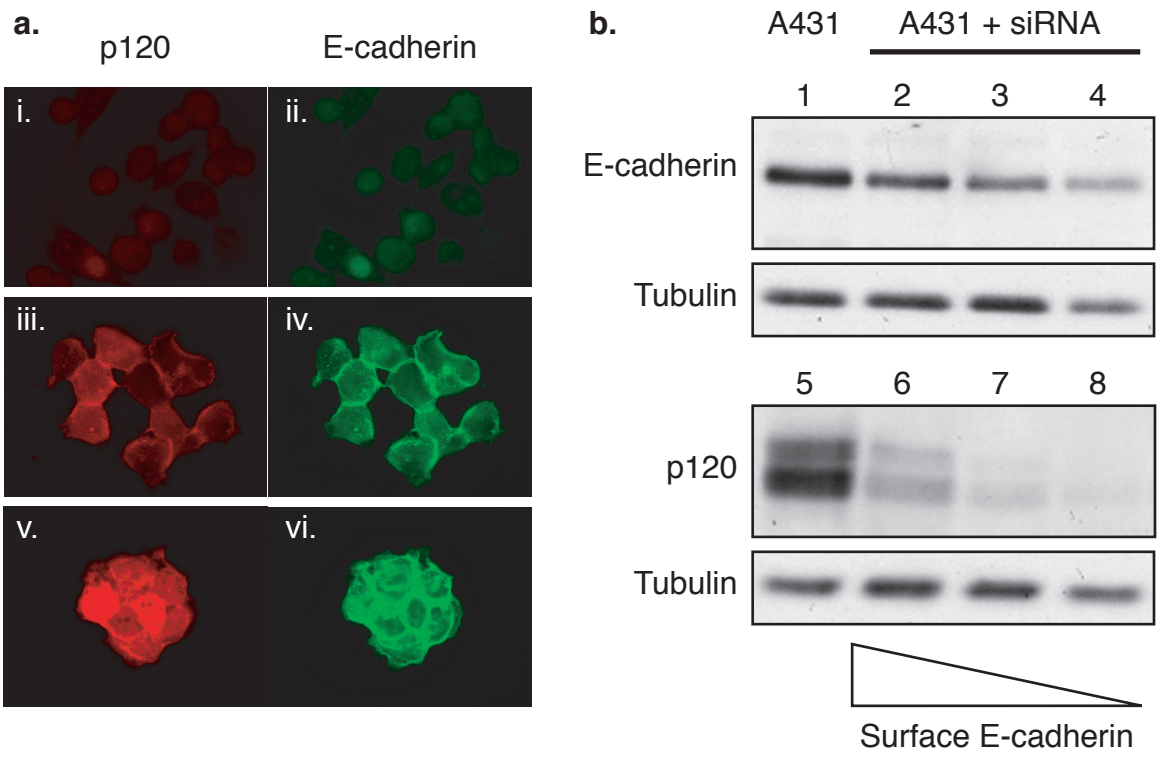


Figure 3. p120 levels act as a set point mechanism for determining cadherin levels. (a) Assay of relationship between p120 and E-cadherin levels by immunofluorescent staining. A431 cells expressing p120 siRNA were infected with the murine p120 r etrovirus and plated sparsely so that individual clones could emerge that expressed widely varying amounts of murine p120. Cells were co-stained by immunofluorescence to examine the p120 – E-cadherin relationship and its affect on cell-cell adhesion. p120 loss (i, ii) caused complete loss of E-cadherin (iii, iv) and the cells were nonadhesive. Intermediate levels of p120 expression (panel iii) permitted intermediate levels of E-cadherin (panel iv), and cell-cell adhesion was partially restored. Higher than normal levels of p120 (panel v) strongly induced E-cadherin (vi) and cell-cell adhesion was robust. These experiments reveal a direct relationship between p120 and E-cadherin levels, and the extent of cell-cell adhesion is directly affected. (b) Quantitative assessment of relationship between p120 and E-cadherin levels. A polyclonal population of cells expressing p120 siRNA was generated by retroviral infection. Individual clones within the population express different levels of p120 depending on integration events that affect the efficiency of the siRNA expression. Using E-cadherin antibodies (HECD-1), the cells were FAC sorted into pools expressing progressively lower levels of E-cadherin. Cell lysates from the samples were split and then Western blotted with anti-p120 (mAb pp120) or anti-E-cadherin (C-20820).

lines (panels v and vi)(see also Fig. 1c). Panels v and vi are overexposed because the common exposure time for the entire panel was chosen to allow better visualization of the low and intermediate p120 levels.

We also quantified the relationship between p120 and E-cadherin expression by FAC-sorting a population of p120 siRNA infected cells with mAb-HECD1, which recognizes the extracellular domain of human E-cadherin (Fig 3b). The cells were sorted into pools with progressively decreasing levels of surface E-cadherin. Cell lysates were generated from each pool, divided in half, and then western blotted for E-cadherin and p120 (Fig. 3b). As in the immunofluorescent assays, the levels of p120 closely paralleled the levels of E-cadherin.

Together, these data show that E-cadherin levels faithfully reflect the level of p120 expression in individual cells, and that the levels of E-cadherin can be experimentally titrated by increasing or decreasing the levels of p120.

p120 family members can functionally substitute for p120

In most epithelial cell lines, p120 is abundant and its close relatives such as ARVCF and δ-catenin are poorly expressed or absent. Although p120 knockdown was sufficient to nearly eliminate E-cadherin in several epithelial cell lines tested, the effect was incomplete in cells such as the colon carcinoma cell line HCT116. An obvious explanation is that p120 family members might partially or completely substitute for p120, depending on their relative abundance. Indeed, HCT116 cells are unusual in that they express moderate levels of ARVCF (not shown). To determine whether other p120 relatives can also regulate E-cadherin turnover, we transiently expressed GFP-labeled

ARCVF or β-catenin in A431 cells that lack p120 as a result of siRNA knockdown (Fig. 4). As a negative control, we also tested plakophilins 3, a more distant p120 relative that binds desmosomal but not classical cadherins. A431 cells expressing p120 siRNA alone were almost completely E-cadherin negative (Fig. 4, panels *i* and *ii*), as described previously, and were not affected by GFP expression (Fig. 4, fluorescent cells in panel i). ARVCF (panel iii) and β-catenin (panel v) localized to adherens junctions and efficiently rescued adhesion by restoring normal E-cadherin levels (panels iv and vi). In contrast, myc-tagged Plakophilin 3 (stained cells in panel vii) did not affect cadherin levels (panel viii) and failed to restore cell-cell contacts. Thus, there is a clear redundant role among close family members with regard to cadherin stabilization, and the occasional significant presence of a p120 family member (e.g., ARVCF in colon HCT116 cells) is likely to account for the fact that E-cadherin loss does not perfectly parallel p120 loss in some cell lines.

Mechanism of E-cadherin-loss

p120 reportedly is the first of the catenins to bind newly synthesized N-cadherin, and co-precipitates with the nascent precursor form of N-cadherin (Wahl et al., 2003). Because of the extraordinary efficiency of E-cadherin destruction after p120 knockdown, we first considered the possibility that p120 binding was necessary to stabilize E-cadherin during or after protein translation and prior to arrival at the cell surface. To examine E-cadherin synthesis in the absence of p120, we labeled the p120-knockdown A431 cells with ^{35}S-methionine and carried out pulse-chase experiments (Fig. 5a). Interestingly, the rate of E-cadherin synthesis was unaffected by the absence of p120 (Fig. 5a, compare top

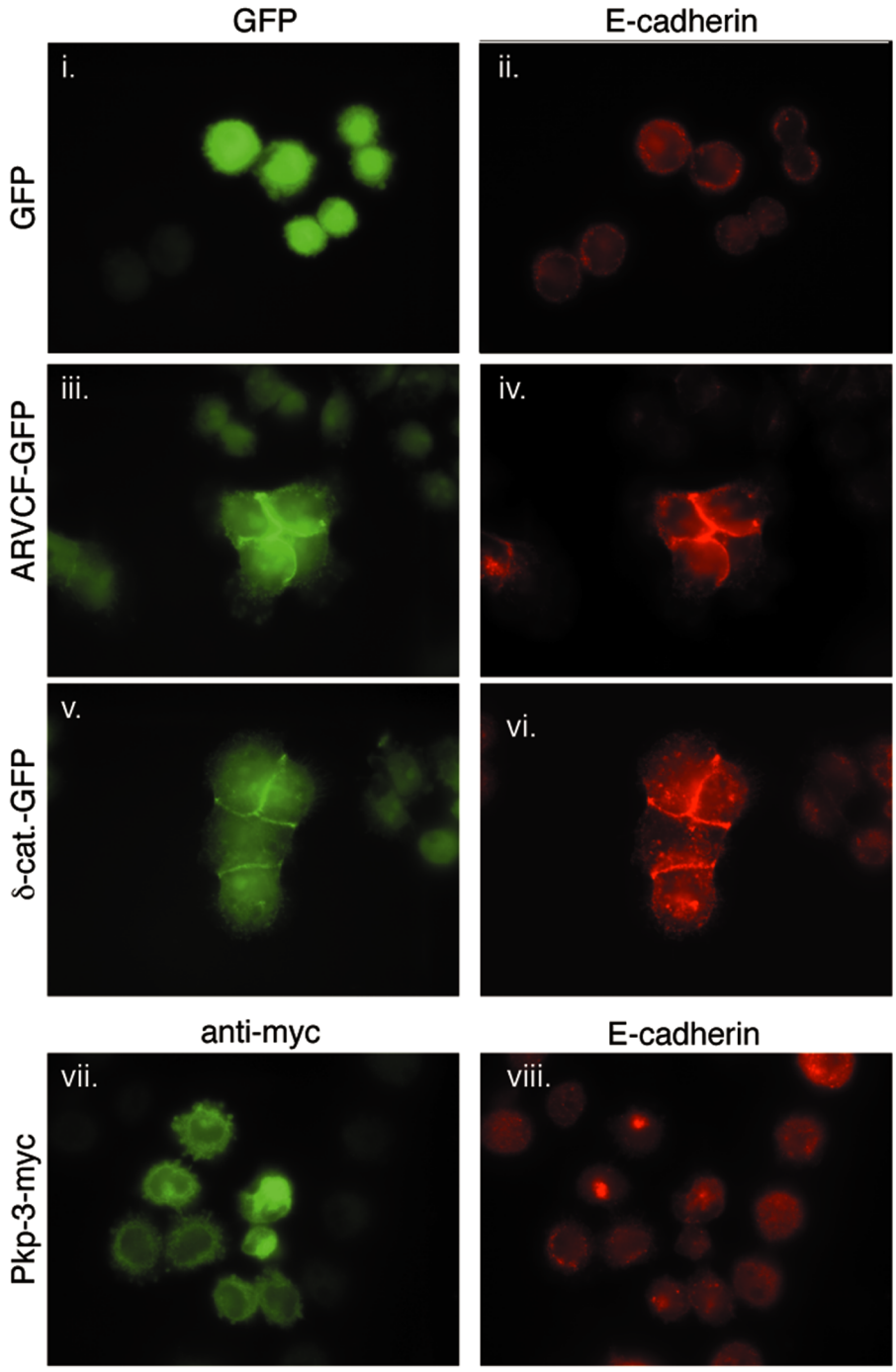

GFP
E-cadherin
i.
ii.
GFP
iii.
iv.
ARVCF-GFP
v.
vi.
δ-cat.-GFP
anti-myc
E-cadherin
vii.
viii.
Pkp-3-myc

Figure 4. Redundant roles for p120 family members ARVCF and δ-catenin. A431 cells stably expressing human p120 siRNA were transiently transfected with ARVCF-GFP, δ–catenin-GFP, or myc tagged plakophilin-3 (Pkp-3-myc). 24 hours post-tranfection, cells were plated sparsely and individual colonies grew for 2 days. Levels of the transfected proteins and E-cadherin were then analyzed by immunofluorescence. GFP expression alone (eluminated cells in panel i) did not affect E-cadherin levels (panel ii). Both ARVCF-GFP (panel iii) and δ–catenin-GFP (panel v) substantially increased levels of E-cadherin (panels iv and vi) and rescued cell-cell adhesion. In contrast, plakophilin 3 (panel vii), a p120-related protein that does not bind classical cadherins, but had no effect on E-cadherin levels (panel viii) or cell-cell adhesion.

panels). Moreover, the processing and turnover of both the precursor and mature forms of E-cadherin were identical for at least an hour following the pulse, after which the cadherin degradation curves diverged rapidly. Degradation of □- and □-catenins paralleled the loss of E-cadherin, as expected from the fact that cadherin-binding stabilizes these catenins.

The fact that the newly synthesized cadherin behaved identically in the presence and absence of p120 for one hour, and until after the precursor form disappeared, suggests that cadherin degradation occurred after arrival at the cell surface. The result was initially surprising because examination of total surface levels of E-cadherin in the p120 knockdown cells (h siRNA), and parental cell lines (Fig. 5b), showed that although surface E-cadherin could be efficiently isolated by biotin labeling and streptavidin pulldown (e.g., lane 2), it was approximately 100 fold less abundant in the p120-deficient cells (Fig. 5b, compare lanes 1 and 2).

To definitively address this issue, we combined the pulse-chase and biotin surface labeling strategies in order to selectively examine the fate of the nascent E-cadherin molecules *vis a vis* their arrival at the cell surface (Fig. 5c). The pulse labeling was conducted as in Fig. 5a, except that surface E-cadherin was subsequently biotin-labeled (as in Fig. 5b) at each time point following the pulse-chase. The surface labeled cadherins were then isolated by streptavidin pulldown, and nascent cadherins were visualized by SDS-PAGE and autoradiography. The data show that the rate of nascent E-cadherin arrival at the cell surface is almost identical in the presence and absence of p120 (Fig. 5c and d, compare parental and siRNA cell lines). The appearance and removal of E-cadherin from the cell surface (Fig. 5c; E-cadherin + streptavidin

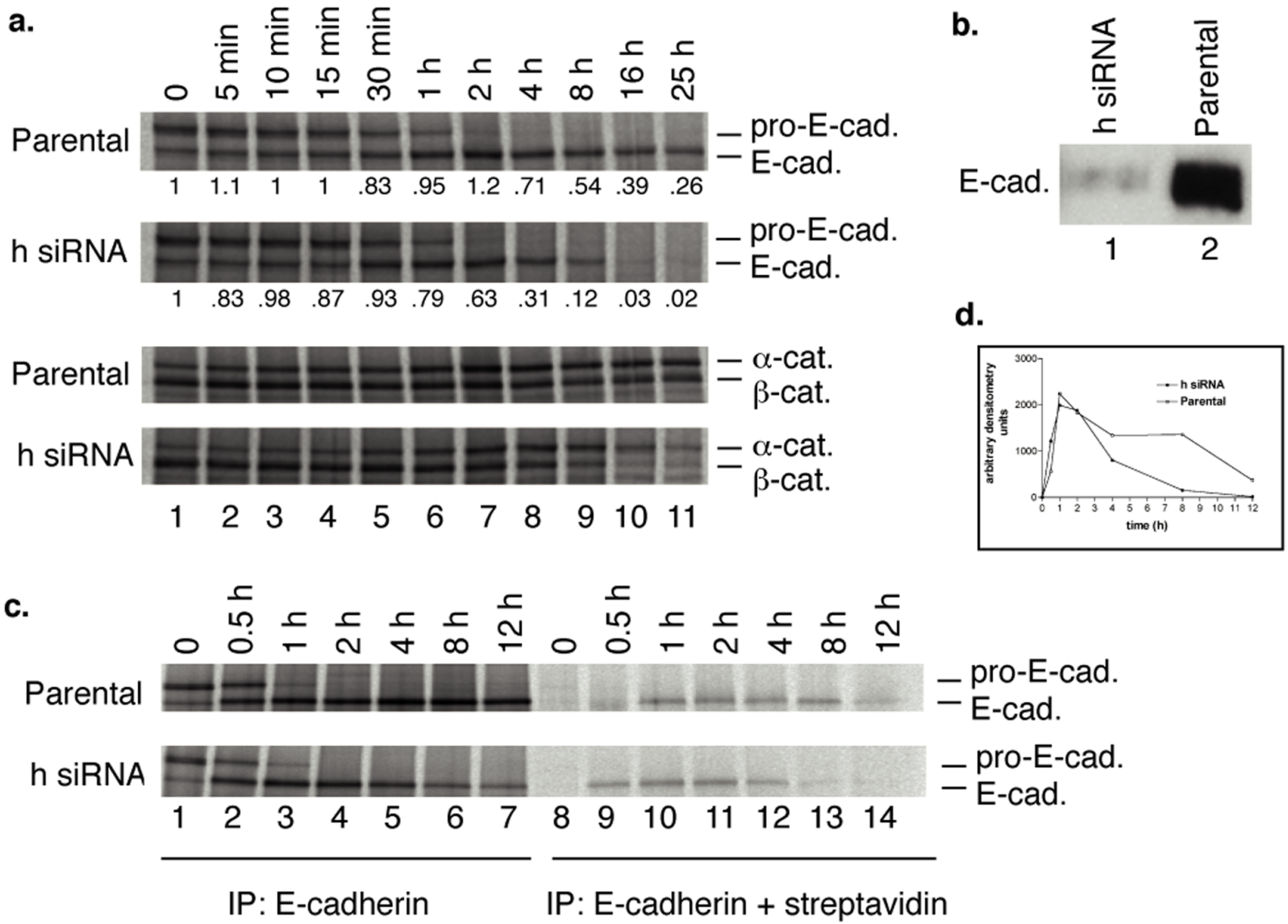

a.
0
5 min
10 min
15 min
30 min
1 h
2 h
4 h
8 h
16 h
25 h
Parental
pro-E-cad.
E-cad.
1 1.1 1 1 .83 .95 1.2 .71 .54 .39 .26
h siRNA
pro-E-cad.
E-cad.
1 .83 .98 .87 .93 .79 .63 .31 .12 .03 .02
Parental
α-cat.
β-cat.
h siRNA
α-cat.
β-cat.
1 2 3 4 5 6 7 8 9 10 11
b.
h siRNA
Parental
E-cad.
1 2
d.
arbitrary densitometry units
3000
2000
1000
0
h siRNA
Parental
0 1 2 3 4 5 6 7 8 9 10 11 12
time (h)
c.
0 0.5 h 1 h 2 h 4 h 8 h 12 h 0 0.5 h 1 h 2 h 4 h 8 h 12 h
Parental
pro-E-cad.
E-cad.
h siRNA
pro-E-cad.
E-cad.
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP: E-cadherin
IP: E-cadherin + streptavidin

Figure 5. p120 regulates E-cadherin turnover at the cell membrane.

(a) E-cadherin synthesis and processing in p120 knockdown cells. E-cadherin turnover was examined by pulse-chase analysis of parental and h p120 siRNA expressing A431 cells. α- and β-catenin processing from the same experiment are shown below. Chase times are indicated across the top. At chase timepoint 0 (15 minutes after initiation of the pulse labeling), E-cadherin synthesis was identical in the presence and absence of p120 (compare E-cadherin bands in panel a). The processing of the pro- (pro-E-cad) and mature (E-cad.) forms were identical for at least 1 hr. Soon thereafter, E-cadherin degradation was significantly accelerated in the absence of p120. (b) Analysis of total E-cadherin surface levels in parental and p120 knockdown A431 cells. The parental and p120 knockdown A431 cells were biotinylated at 4 °C for 20 min to label surface cadherins. To specifically measure the surface levels, E-cadherin was first immunoprecipitated directly with E-cadherin mAb HECD-1. The sample was eluted with 0.5% SDS and then re-precipitated with streptavidin-coated beads to isolate the surface-labeled pool. E-cadherin levels at the surface in p120 knockdown cells (lane 1) are at least 100 fold diminished relative to the parental cells (lane 2). The result in lane 2 shows that the surface E-cadherin can be efficiently labeled (and detected) by this method. (c) Tracking the arrival of newly synthesized E-cadherin to the cell surface. The methods in (a) and (b) were combined to determine whether newly synthesized E-cadherin could transit to the cell surface in the absence of p120. The results in panel (c) were quantified by densitometry and represented graphically in panel (d). Parental and p120 knockdown (h siRNA) cells were labeled with ^{35}S-methionine for 15 min, chased at 37° C for the times indicated across top, and placed on ice (4° C) to suspend trafficking. Cell surface proteins were immediately biotinylated at 4° C for 20 min as in (b). Surface E-cadherin was then isolated as in (b), and the nascent (^{35}S-methionine labeled) E-cadherin pool was visualized by SDS-PAGE and radiography. Nascent E-cadherin appeared at the surface at 30 min and peaked at 1 hr. The absence of p120 had no effect on this result. Therefore, p120 is not required for E-cadherin synthesis or trafficking, but is essential to regulate E-cadherin turnover soon after its arrival at the cell surface.

immunoprecipitations) are quantified by densitometry and displayed graphically in Fig. 5d. Note that peak levels of nascent (^{35}S-labeled) E-cadherin at the cell surface occurred at 1 hour, and by 4 hours, the nascent cadherin was either moving off the surface or getting degraded. The timing is consistent with the 4 hour time point in Fig. 5a, which marks the first interval where degradation of the unbound cadherin sharply accelerates. Clearly, E-cadherin transits normally to the surface in the absence of p120, but is then rapidly turned over.

To identify the mechanism of degradation, we treated p120-knockdown cells with over 30 agents known to inhibit factors that have been reported to affect cadherin stability and turnover. Examples include inhibitors of Presenilin-1, caspases, metalloproteinases, and calpain. Cells were incubated for 24 hours with predetermined amounts of the various inhibitors, and then analyzed by immunofluorescence (not shown) or Western blotting for changes in levels of E-cadherin (Fig. 6). While the majority of the inhibitors had no affect, several proteosome inhibitors (i.e., PS341, lactacystin, ALLN, and MG132), significantly blocked E-cadherin degradation (Fig. 6; lactacystin, lanes 1 and 2; PS341 lanes 3 and 4). The reduced amount of E-cadherin at the higher PS341 dose (compare lanes 3 and 4) reflects toxicity of this compound. Of the two commonly used lysosomal inhibitors we tried, ammonium chloride (NH_4Cl, lane 5) had no effect, but chloroquine (Chl, lane 6) blocked E-cadherin degradation almost as effectively as the proteosome inhibitors. In both the PS341 and chloroquine treated cells, cytoplasmic pools of E-cadherin increased, but the increased levels were not reflected by increased adhesion or higher surface cadherin levels. Thus, these inhibitors appear to block cadherin degradation but do not affect internalization. The data suggest that when newly

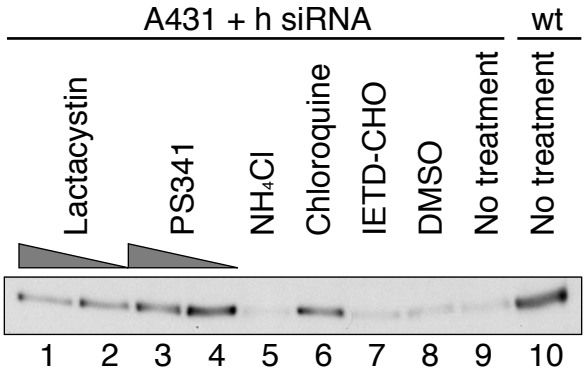


Figure 6. Mechanism of E-cadherin degradation.

The effects of various inhibitors known to influence cadherin stability were assayed in the stable p120 knockdown A431 cells. E-cadherin levels from samples treated for 24 hrs (lanes 1 – 9) were monitored by Western blotting whole cell lysates and compared to normal E-cadherin levels in the parental cell line (lane 10). Inhibitor concentrations were: lactacystin 10 μM (lane 1), 3.3 μM (lane 2); PS341 100 nM (lane 3), 33 nM (lane 4), ammonium chloride 5mM (lane 5), Chloroquine 33μM (lane 6), and IETD-CHO 10nM (lane 7). DMSO is the control condition (lane 8). The proteosome inhibitors lactacystin and PS341 increased E-cadherin levels (compare lanes 1 - 4 to lanes 8 and 9). The lower cadherin levels after 100 nM PS341 (lane 3) relative to the 33 nM treatment (lane 4) is due to toxicity at the higher concentration. Of the lysosomal inhibitors, chloroquine (lane 6) but not ammonium chloride (lane 5) increased E-cadherin levels. A Caspase 8 inhibitor (lane 7) that has been shown to inhibit E-cadherin degradation in myeloma cells had no effect.

synthesized E-cadherin arrives at the cell surface, p120 is required to prevent the immediate targeting of unbound E-cadherin for degradation by the proteosome and/or lysosome. We conclude that p120 regulates cadherin turnover by controlling either internalization, or possibly an immediately subsequent decision whereby internalized cadherins are sorted into recycling or degradation pathways.

Discussion

Here, we provide evidence that the core function of p120 in cadherin complexes is to regulate cadherin turnover. We showed previously that the stabilizing effect of p120 on E-cadherin in a p120-deficient SW48 cell line involved a post-transcriptional mechanism and required direct p120-E-cadherin interaction (Ireton et al., 2002a). However, it was not clear whether this phenomenon was generally applicable beyond SW48 cells, nor could we determine the underlying mechanism. Here, using siRNA and/or p120 reconstitution, we show that E-cadherin levels depend absolutely on p120 expression. Importantly, this set point mechanism is common to other (probably all) p120-binding cadherins since p120 knockdown also induced significant downregulation of P-, VE- and N-cadherins. The timing and location of p120 action argue strongly that p120 regulates adhesion via controlling cadherin turnover at the cell surface. These observations have crucial implications for roles of p120 in cadherin function and cancer.

We believe that the only exception to the requirement for p120 occurs in cells that express p120 family members such as ARVCF or δ-catenin. This qualifier is based in part on cell lines such as HCT116 where the observed reduction in E-cadherin levels after p120 siRNA expression did not perfectly parallel the extent of p120 loss. Indeed,

although ARVCF is typically difficult to detect in many epithelial cell lines, it is expressed at moderate levels in HCT116 cells (not shown). Our data show that ARVCF and β-catenin efficiently compensate for p120 loss when ectopically expressed in A431 cell lines expressing p120 siRNA. Despite significant structural and sequence similarity, plakophilin 3 had no effect, presumably because it does not bind classical cadherins. These data strongly imply that surface cadherin stability is invariably dependent on the binding of either p120 or a closely related family member, and the presence of variable levels of p120 family members likely accounts for the discrepancy in cell lines where p120 knockdown does not cause a corresponding loss of resident classical cadherins.

The fact that p120 availability limits cadherin levels has several crucial implications. For example, overexpression of dominant negative (DN) cadherins frequently downregulate expression of endogenous cadherins (Dong et al., 1996; Fujimori and Takeichi, 1993; Kintner, 1992) but the mechanism is unknown. Our data strongly suggest that a key action of DN-cadherins is the sequestering of endogenous p120, thereby driving the turnover and degradation of endogenous cadherins. In addition, cadherin levels in cells may ultimately be controlled by factors that regulate p120 levels, and competition for interaction with p120 is likely to be physiologically relevant in cells that express more than one cadherin.

In theory, the absence of cadherins in p120-deficient cells indicates either a failure to normally synthesize cadherins, or an efficient means of eliminating them when p120 is not present. However, our pulse-chase data indicate that p120 is not required for normal synthesis or transit of cadherin to the cell surface. Instead, p120 absence dramatically accelerates cadherin degradation after its arrival at the surface, indicating a

role in regulating cadherin turnover at the membrane (modeled in Fig. 7). Our data does not precisely distinguish the point at which p120 acts to prevent degradation. The simplest explanation is that p120 limits degradation by regulating internalization. Only cadherin-bound p120 is phosphorylated (Thoreson et al., 2000), and p120 phosphorylation is the most likely means of regulating p120-cadherin affinity and/or p120 activity in the complex. We cannot rule out the less likely possibility that once internalized, p120 might control the next step, which targets the endocytosed cadherin for either degradation or recycling back to the surface. Regardless, it is likely that the ultimate destruction of the cadherin in p120-deficient A431 cells resides mainly in the proteosome, and to some extent in the lysosome.

Under normal circumstances, cadherin turnover is constitutive and endocytosis is a crucial mechanism for downregulating cadherin adhesiveness (Le et al., 2002; Le et al., 1999; Xiao et al., 2003). We postulated previously that p120 acts as a switch, inducing the assembly or disassembly of cadherin complexes through transient signaling events (probably tyrosine and serine phosphorylation), which in turn might regulate cadherin clustering. Our new data strongly favors a mechanism whereby dynamic assembly and disassembly of cadherin complexes is driven primarily by regulation of cadherin turnover rather than physical clustering (Fig. 7). A plausible explanation is that the rate of cadherin turnover is dictated by events at the cell surface that transiently increase or decrease p120 affinity for cadherins. The off state favors internalization/degradation, whereas the on state favors retention/recycling. The low affinity of p120 for cadherins, as judged by co-immunoprecipitation experiments (Thoreson et al., 2000), probably reflects the ability of p120 to rapidly alternate between cadherin bound and unbound

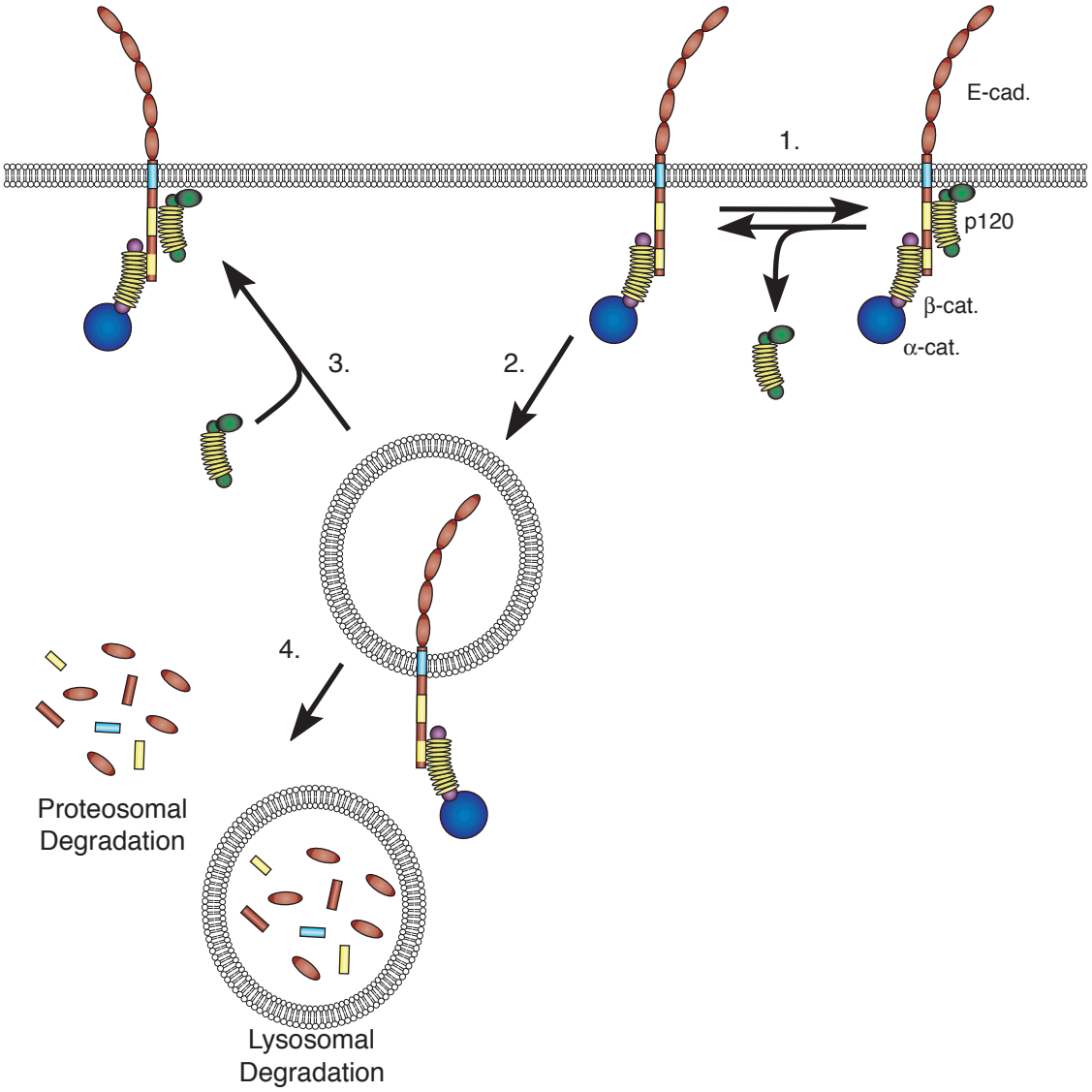

E-cad.
1.
p120
β-cat.
α-cat.
2.
3.
4.
Proteosomal
Degradation
Lysosomal
Degradation

Figure 7. Model for p120 function in regulating cadherin turnover.
The low affinity of p120 for cadherins, as judged by coimmunoprecipitation experiments, probably reflects the ability of p120 to rapidly alternate between cadherin-bound and -unbound states. (1.) Our data suggests that the rate of cadherin turnover is controlled by cell surface events that transiently increase or decrease p120 affinity for cadherins. Thus, cadherin complexes exist in a dynamic equilibrium between p120-bound and unbound states, which in turn may be regulated by p120 phosphorylation (not shown). (2.) Unbound cadherin is targeted for internalization, possibly via a Hakai-like ubiquitination mechanism (see discussion). (3.) We cannot yet rule out an alternative pathway where p120-binding is irrelevant for internalization but mediates a sorting decision that recycles internalized cadherin back to the membrane. (4.) Regardless of the exact decision point, unbound cadherin is targeted for degradation by the proteosome and/or lysosome. Considerable evidence indicates that signaling events at the cell surface modulate phosphorylation of the cadherin-bound pool of p120. The simplest interpretation of these observations is that p120 phosphorylation regulates its steady state affinity for cadherins, which in turn regulates adhesion by controlling the rate of cadherin turnover. Note that α– and β–catenin are passive players in this model. They likely participate in clustering and certainly mediate the cytoskeletal interaction (not shown), but their role may be secondary to regulating surface cadherin levels, which is almost completely determined by p120.

states. It is worth noting that □- and □-catenins are largely passive players in this model. Because cadherin binding controls their stability, their fate is ultimately tied to cadherin levels, which are clearly controlled by p120. Of course, turnover and clustering are not mutually exclusive mechanisms, but our current data suggests that turnover may take precedence.

Recent experiments in *C. elegans* and *drosophila* indicate that p120 is not essential in these organisms. Indeed, both worms (Pettitt et al., 2003) and flies (Myster et al., 2003) are viable when p120 is removed, and p120-uncoupled E-cadherin can substitute effectively for wild type E-cadherin in flies (Pacquelet et al., 2003). In contrast, the murine p120 knockout is embryonic lethal (Walter Birchmeier and Al Reynolds, unpublished observations). Additionally, our current data show clearly that p120 is essential in mammalian cells. It is possible that mammalian p120 has evolved both additional family members and increased complexity to accommodate the developmental demands of higher organisms.

An unanswered question is the exact targeting mechanism for internalization and/or degradation of cadherins not associated with p120. Because direct binding of p120 to E-cadherin is required, it is possible that p120 binding blocks the interaction of an unknown binding partner (or event) that targets E-cadherin for degradation. Candidates include presenilin-1 (Baki et al., 2001; Marambaud et al., 2002) and Hakai (Fujita et al., 2002), which are reported to compete with p120 for binding the cadherin juxtamembrane domain. Presenilin-1 binding promotes proteolytic degradation of E-cadherin (Baki et al., 2001; Marambaud et al., 2002), whereas Hakai is a ubiquitin ligase that binds tyrosine phosphorylated E-cadherin leading to its ubiquitination and

destruction (Fujita et al., 2002). Several tyrosine kinase receptors are turned over via a similar mechanism involving the oncogene and ubiquitin ligase Cbl, which binds tyrosine phosphorylated residues via its classical SH2 domain (reviewed in Hicke, 1999). However, we were unable to block E-cadherin destruction in the p120 siRNA cell lines with either presenilin or tyrosine kinase inhibitors (data not shown). Moreover, the mechanism we describe is common to several cadherins, whereas the Hakai mechanism appears specific for E-cadherin. Nonetheless, our data favor a model where an E-cadherin targeting event is triggered by the absence or transient off-loading of p120.

Finally, several lines of evidence suggest that this new role for p120 in regulating cadherin turnover may be important in cancer. In cell lines, E-cadherin loss leaves p120 stranded in the cytoplasm but has little effect on p120 levels. It is well established that E-cadherin loss occurs frequently by mutation (Berx et al., 1998) and by epigenetic mechanisms (Comijn et al., 2001; Matsumura et al., 2001) that probably do not involve p120. In contrast, p120 loss clearly represents a different scenario which directly induces loss of E-cadherin and thus ultimately the entire cadherin complex. It follows that p120 loss may precede cadherin loss in the reported subset of tumors that have been shown to lack both proteins (reviewed in Thoreson and Reynolds, 2002). Accumulating evidence suggests that p120 downregulation occurs frequently in colon, prostate, lung, bladder, breast, and several other malignancies (reviewed in Thoreson and Reynolds, 2002). p120 is both mutated and under-expressed in the colon carcinoma cell line SW48, and indeed, E-cadherin is indeed strongly downregulated in these cells providing the first physiologically relevant example of this phenomena in a carcinoma cell line. However, no other p120-deficient cell lines have been described, and physical alterations in the

p120 gene locus have not been associated with malignancy. Together, these observations suggest that p120 downregulation in tumors occurs by an epigenetic mechanism that has yet to be identified, and raise the possibility that like E-cadherin, p120 acts as a tumor suppressor.

In conclusion, we show that p120 levels determine steady state levels of functional cadherins by regulating cadherin turnover at the cell surface. This is likely the core function of p120 in the cadherin complex and suggests that cadherin adhesiveness is modulated, in part, by signaling events that dynamically influence p120 – cadherin affinity. In addition, p120 is clearly at the top of the cadherin "food chain" in terms of who controls the overall fate of the complex. Together with reports of p120 downregulation in a wide range of epithelial tumors, these data suggest a role for p120 as a tumor suppressor.

CHAPTER IV

P120 IS ESSENTIAL FOR EPITHELIAL MORPHOGENESIS

Introduction

The cell-cell adhesion molecule E-cadherin, is essential in establishing epithelial morphology. It is required to establish polarized epithelial barriers, and without it, embryos are incapable of forming the first differentiated epithelium (Larue et al., 1994). Moreover, loss of E-cadherin is a key event in the transition towards metastasis, since E-cadherin is frequently lost in a wide range of metastatic tumors and its loss in benign adenomas correlates with a transition to invasion (Cavallaro and Christofori, 2004; Nollet et al., 2000). Thus, E-cadherin is considered a master regulator of the epithelial phenotype.

The catenins are cytoplasmic binding partners of E-cadherin that are required to dynamically regulate E-cadherin function (Yap et al., 1997a). □-catenin binds to the C-terminus of E-cadherin (Nagafuchi and Takeichi, 1989; Ozawa et al., 1990; Stappert and Kemler, 1994) and □-catenin binds both □-catenin and actin, thereby linking E-cadherin to the actin cytoskeleton (Aberle et al., 1994; Hulsken et al., 1994). In contrast, p120 catenin (p120 hereafter) binds the juxtamembrane domain of E-cadherin (Thoreson et al., 2000; Yap et al., 1998), and while p120 does not physically interact with the actin cytoskeleton, it may functionally interact with actin through the regulation of the Rho GTPases (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). We have recently shown *in vitro* that p120 is required, in a dose-dependent manner, to stabilize the

cell-surface expression of E-cadherin (Davis et al., 2003b). Interestingly p120 loss correlates with E-cadherin loss in cases of inflammatory bowel disease (IBD) (Karayiannakis et al., 1998) and in a wide range of tumors, such as in breast, lung and pancreas (Thoreson and Reynolds, 2002). Finally, *in vivo* expression of dominant negative cadherins, which sequester p120 and downregulate endogenous E-cadherin, cause IBD-like phenotypes and adenomas (Hermiston and Gordon, 1995). Together, these data strongly suggest that p120 is an important tumor modifier through its regulation of E-cadherin, however a direct *in vivo* role for p120 in E-cadherin stabilization and tumor progression has not been shown.

To directly test the effect of p120 loss on E-cadherin stability *in vivo* and the effects of p120 loss on tumor progression, we attempted to target the deletion of p120 to the mammary gland using MMTV-Cre. MMTV-Cre deletion of p120 should circumvent embryonic lethality associated with a complete loss of p120 (personal communication from Walter Birchmeier) and has been successfully used to study essential genes such as E-cadherin (Boussadia et al., 2002), Brca1 (Xu et al., 1999), and Bclx(Wagner et al., 2000). However, MMTV-Cre deletion of p120 results in neonatal lethality, with a complete loss of p120 in the salivary glands and lachrymal glands and a mosaic loss of p120 in the skin. Like the mammary gland, the salivary gland is a branched epithelial secretory structure. Salivary morphogenesis occurs during a short window between e12.5 and parturition (Melnick and Jaskoll, 2000), and the events regulating its development are remarkably similar to other well-studied branched organs like the lung and pancreas. Thus, despite an inability to examine the role of p120 in tumor progression, conclusions

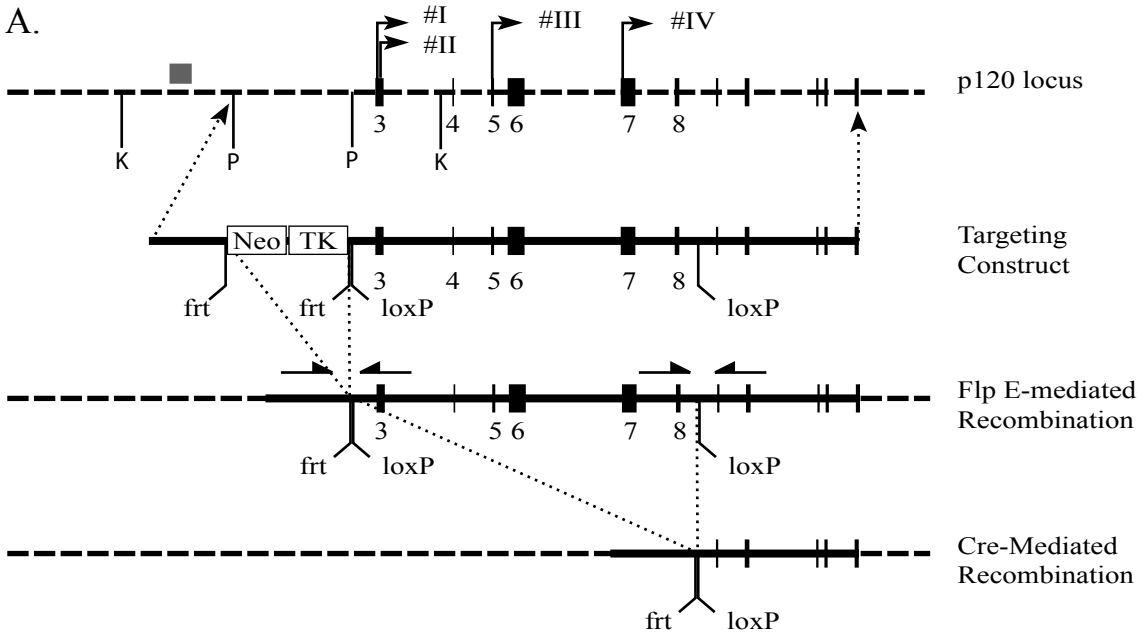
drawn in the embryonic and neonatal salivary gland will likely apply to these other tissues.

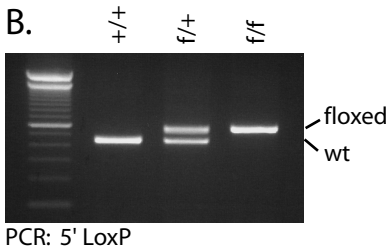
We report that p120 is essential for E-cadherin stability *in vivo*, and we show that p120 is required for acinar development and proper ductal morphogenesis. Consistent with a role for p120 in inflammatory diseases such as IBD and pancreatitis, we observe indicators of chronic inflammation in p120 knockout mice as early as e16.5. Finally, despite the young age of knockout animals, knockout salivary glands bear many hallmarks of precancerous lesions: including ductal hyperplasias and papillary structures with loss of polarity, nuclear crowding, and pseudostratification. Although, reactive changes due to inflammation can mimic early precancerous lesions, these phenotypes are observed prior to and are developed beyond that which is caused by inflammation. While these data are unable to demonstrate a role for p120 in tumor progression, the conclusive need for p120 in regulating E-cadherin levels and the presence of lesions bearing neoplastic hallmarks strengthen our hypothesis that p120 likely plays a role in tumor progression.

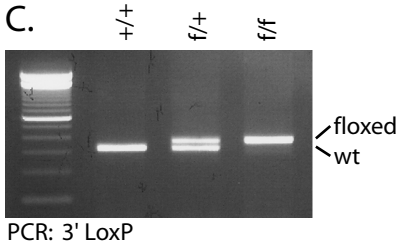
Results

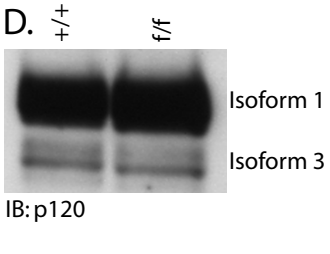
Conditional knockout of p120.

To study the role of p120 *in vivo*, we generated a conditional p120 knockout in mice using Cre/lox technology. To generate a targeting construct (Fig. 1A) for the conditional removal of p120, the murine p120 gene was isolated from the RPCI-22 (129S6/SvEvTac) Mouse BAC Library (Osoegawa 2000), and we sequenced a 20 kb









E.

Age	Genotypes generated from (f/f) X (f/+, Cre)				n	p
	(f/f, Cre)	(f/+, Cre)	(f/f)	(f/+)		
Parturition	29%	21%	24%	26%	100	0.715
Weaning	2%	33%	27%	37%	100	>0.001
Expected	*25%*	*25%*	*25%*	*25%*		

Figure 1. MMTV-Cre deletion of p120 is postnatally lethal.
(A) The targeting construct was designed to remove the four translational start sites (I-IV) of p120. A selection cassette, flanked by frt sites, and a downstream LoxP site were inserted into intron 2 and a second LoxP site was placed intron 8. The selection cassette was removed by FlpE-mediated recombination of the frt sites in vitro, and the four start sites were knocked out by MMTV-Cre-mediated excision in vivo. (B & C) PCR genotyping of wild type (+/+), heterozygous floxed-p120 (f/+), and homozygous floxed-p120 mice amplifies a sequence containing the 5' (B) or the 3' (C) LoxP sites. (D) Lysates from murine embryonic fibroblasts of wild type and homozygous floxed-p120 mice were immunoblotted for p120. Importantly, insertion of LoxP sites into intron 2 and 8 has no effect on p120 expression or alternative splicing. (E) Homozygous floxed mice were crossed with heterozygous mice that bear the MMTV-Cre transgene. Four progeny genotypes were expected in equal proportions. However, only 2% of the population were knockout animals (f/f, Cre) at weening, whereas, all genotypes were observed in proper Mendelian ratios at parturition. Half arrows: primers used in PCR-based genotyping; Black boxes: exons; Grey box: probe used in screening for homologous recombination; K: KpnI; P: PshAI; Neo: Neomycin resistance gene; TK: Thymidine kinase gene.

region spanning part of intron 2 through exon 15. To avoid truncation mutants generated from ATG's in downstream exons, LoxP sites were placed in introns 2 and 8, and a Neo-TK selection cassette, flanked by frt sites, was placed immediately upstream of the 5' LoxP site in intron 2.

To produce p120-floxed animals, we transfected our targeting construct into 129SvEvTac ES cells and obtained six G418 resistant clones with a homologous integration of the targeting construct (data not shown). Subsequent transfection with FlpE recombinase yielded three Gancyclovir resistant clones that lack the Neo-TK selection cassette. Using standard techniques, we generated chimeric mice from one of these clones, and the mice were then bred for germline transmission of the floxed allele.

Homozygous p120-floxed animals displayed no overt phenotypes, suggesting our manipulations had no effect on p120 protein production. This was verified by western blot analysis of wild type (+/+) and homozygous p120 floxed (f/f) embryonic fibroblasts, which showed no differences in p120 levels or isoform stoichiometry (Fig. 1D).

MMTV-Cre-mediated deletion of p120 is lethal.

We targeted the deletion of p120 to the mammary gland using MMTV-Cre (generated by Kay Wagner and Lothar Hennighausen and obtained from the Mouse Models of Human Cancer Consortium) with the intention of examining the role of p120 in tumor progression. We bred homozygous floxed-p120 mice (f/f) with heterozygous mice also carrying the MMTV-Cre recombinase (f/+, Cre). Based on this breeding strategy, we expected 25% of each litter to be of knockout genotype, homozygous floxed-p120 with the Cre recombinase (f/f, Cre). However only 2% of the genotyped weanlings

were knockouts, suggesting that the knockout animals died prior to weaning. Therefore, we examined animals at e12.5, e18.5, and parturition to determine if knockouts died *in utero* or postpartum. Mendelian ratios were observed at parturition, indicating that mutant animals died postpartum. Furthermore, nearly all knockout animals died within one to two days of birth (not shown). Given the effectiveness of studying MMTV-Cre-mediated deletion of essential genes such as E-cadherin (Boussadia et al., 2002), Brca1 (Xu et al., 1999), and Bclx(Wagner et al., 2000), we were surprised that MMTV-Cre-mediated deletion of p120 resulted in neonatal lethality (Figure 1E); the cause of death was unclear.

p120 is essential for proper branching morphogenesis and acinar development.

MMTV-Cre is expressed in multiple tissues (Wagner et al., 1997), and neonatal mutants showed near complete loss of p120 in lachrymal glands and the major and minor salivary glands and showed mosaic loss of p120 in the epidermis and hair follicles. p120 loss was not seen in any other organ (not shown). While minor defects were observed in hair follicle maturation and lachrymal gland development (not shown), severe defects were seen in the submandibular and sublingual salivary glands.

Immature salivary glands begin as an invagination of the oral epithelium as early as e11.5 to form the initial bud (Jaskoll and Melnick, 1999) (Melnick and Jaskoll, 2000). Successive rounds of cleavage and extension of the end buds lead to formation of the ductal tree (Spooner et al., 1989). The ducts branch and narrow as they extend distally, and they transition from bilaminate structures to ones with a single layer of cuboidal cells as they approach the termini of the mature tree. Beginning at e16.5-e17.5, cells at the

distal tips of the ducts differentiate to form columnar cells of the secretory acini (Figure 3D). These are characterized in part by basally located nuclei and expression of mucin . By parturition, salivary gland development is nearly complete.

MMTV-Cre deletion of p120 in ductal cells of submandibular and sublingual salivary glands occurred by e13.5, as p120 immunostaining was subtly reduced compared to wild type (Fig. 2A & C), but no morphological differences were observed by histological analysis of H&E stained tissue sections (Fig. 3A & E). By e14.5, p120 protein levels were clearly depleted (Fig. 2D) in knockout animals, and there were striking differences between wild type and mutant animals (Fig. 3B & F). Although the first round of branching proceeded normally in mutant glands, lumina of e14.5 ducts were obviously dilated, as compared to wild type (see Fig. 3B & 6G). Small ductal masses and cells that were apparently free-floating were observed within the dilated lumina, and the end buds of mutant salivary glands were misshapen compared to the smooth, rounded end buds of wild type glands.

By e15.5 (Fig. 3C & G), the second round of extension and branching had occurred. Unlike normal ducts, mutant ducts followed a tortuous path, branched irregularly, and contained widespread ductal outgrowths. Furthermore, while the end buds of wild type glands were well formed and bulbous, end buds of mutant glands were abnormally shaped and appeared as extensions of the ducts. Finally, ductal outgrowths were larger than those seen in e14.5 mutant glands (arrow, Fig. 3G).

At e17.5, mutant glands were visibly smaller and lacked the multitude of acinar structures (Fig. 3H) that were readily identifiable in wild type glands (arrowhead, Fig.

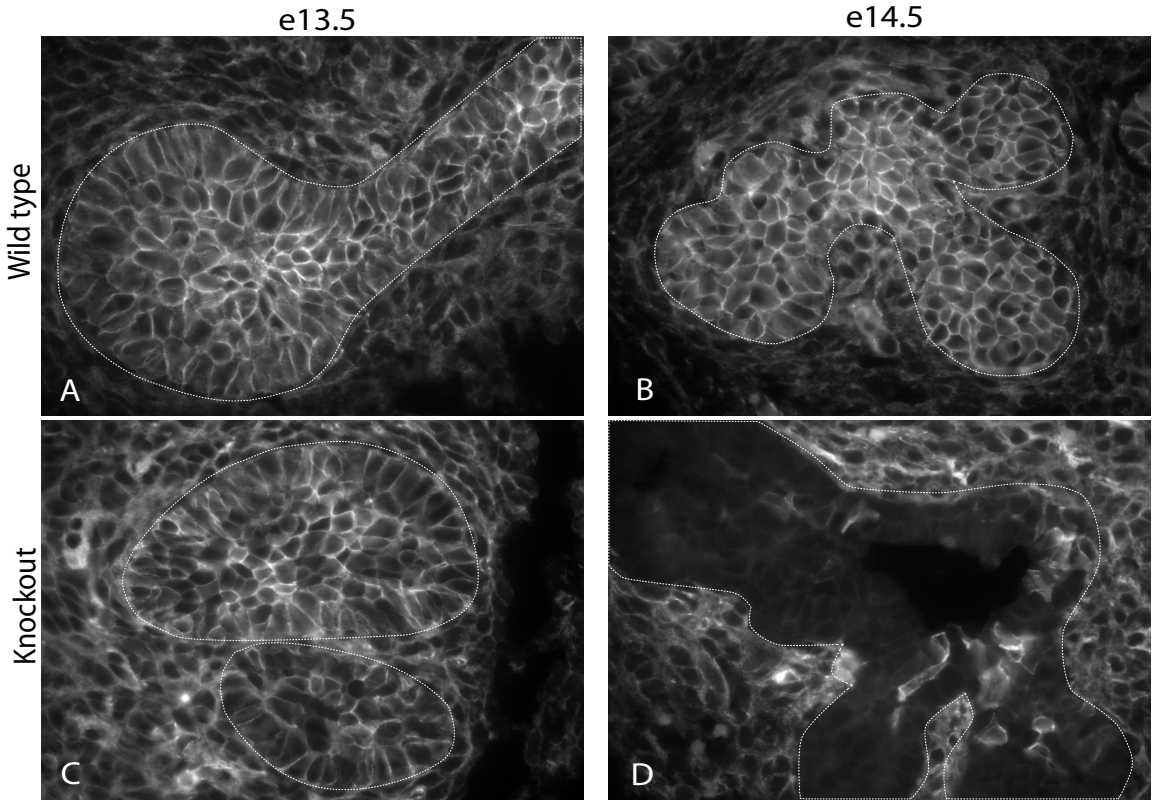


Figure 2. MMTV-deletion of p120 leads to p120 loss in submandibular and sublingual salivary glands by e14.5. p120-specific immunostaining of e13.5 (A & C) and e14.5 (B & D) salivary glands demonstrated that p120 expression is lost by e14.5 in mutant animals. Compare (C) to (D). The dotted line in each panel demarcates the epithelium.

e13.5
e14.5
e15.5
e17.5
Wild type
Knockout
A
B
C
D
E
F
G
H

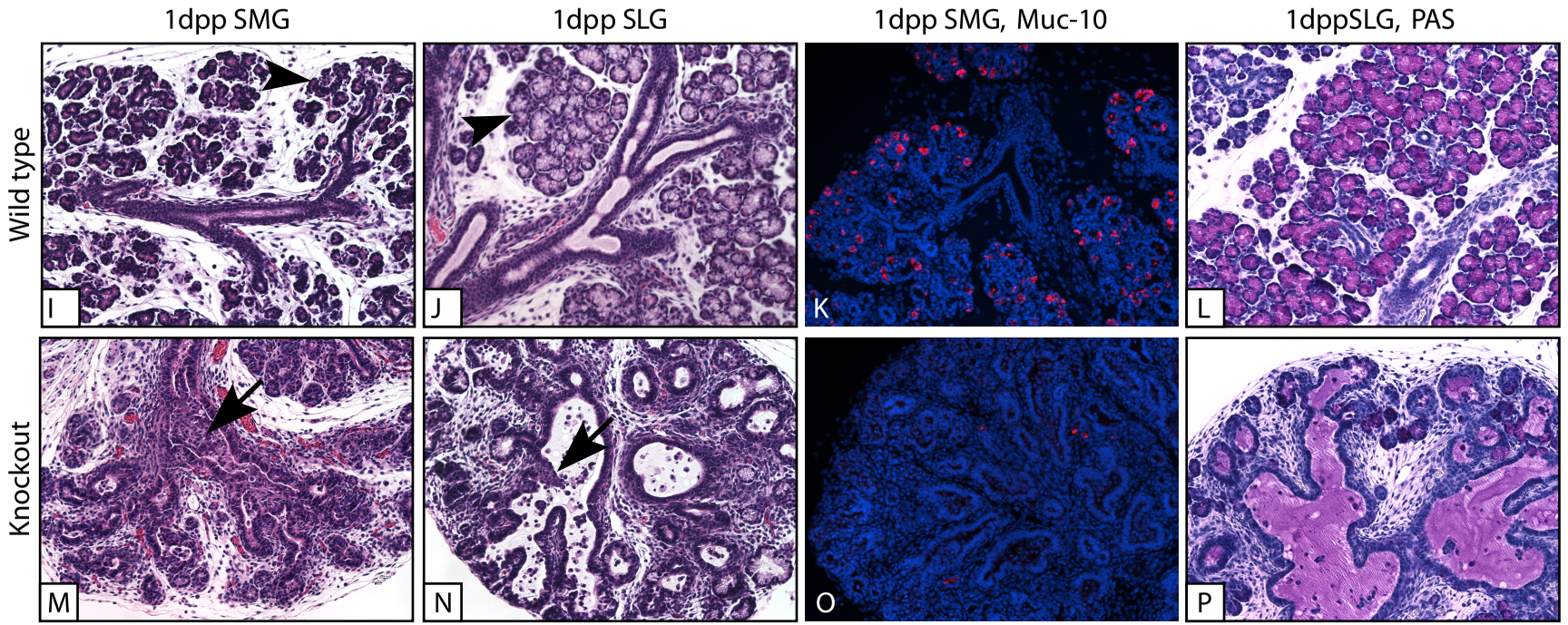


Figure 3. p120 is essential for ductal morphogenesis and acinar development in the murine salivary gland and p120 loss causes abnormal lumen dilation and ductal outgrowths. H&E stained wild type and mutant salivary sections from e13.5 (A & E), e14.5 (B & F), e15.5 (C & G), e17.5 (D & H), and 1day postpartum (I - P) shows that p120 loss caused severe developmental defects. These include ductal dilation as early as e14.5 (F), tortuous ductal extension (G), lack of acini at e17.5 (H), ductal occlusion by widespread outgrowths in 1dpp submandibular glands (M), and cystic dilation of 1dpp sublingual glands (N). Lack of acini in 1dpp animals was confirmed in the submandibular glands by embryonic MUC-10 immunostaining (K & O) and in the sublingual gland by PAS staining (L & P). Note the mucin-positive staining of the mutant sublingual ducts. SMG: submandibular gland, SLG: sublingual gland, 1dpp: one day postpartum, H&E: hematoxylin and eosin, arrowheads: acini, arrows: ductal outgrowths.

3D). Furthermore, ductal hyperplasias obviously displaced surrounding tissues (arrow, Fig. 3H), giving the appearance that these masses were continuing to grow.

Three days later, at parturition, mutant glands still lacked acinar structures (Fig. 3M-N vs. arrowheads in Fig. 3I-J). To verify that acinar differentiation was either inhibited or blocked in mutant mice, we stained salivary sections for mucin, which is a marker of terminal differentiation in the salivary glands. As the submandibular and sublingual express different mucins, submandibular glands were stained with embryonic Muc-10 (Melnick et al., 2001)(Fig. 3K & O), and sublingual glands were stained with PAS (Fig. 3L & P), a common stain that renders mucin pink. Mutant submandibular glands either completely lacked embryonic Muc-10 or had greatly reduced levels depending on the degree of p120 loss (Fig. 3O). Sublingual salivary glands, on the other hand, do not express embryonic MUC-10 but express other forms of mucin. Therefore their acini stained strongly pink by PAS. Examination of mutant sublingual glands showed little to no mucin positive acini, but instead showed strong mucin staining in the dilated ducts (Fig. 3P). These results indicate that acinar differentiation is indeed inhibited or altogether prevented by p120 loss. In addition, while both submandibular and sublingual ducts of knockout animals were similarly dilated at earlier time points, 1 day postpartum (1dpp) lumina of mutant sublingual glands are cystic and those in the minor ducts of the submandibular gland are nearly occluded by ductal outgrowths and shed cells (Fig. 3M & N, respectively).

Finally, high magnification examination of day old mutant glands revealed interesting abnormalities in ductal architecture. As noted previously, wild type ducts have bilaminar architecture, with rounded evenly-spaced nuclei (Fig. 4A-B). In contrast,

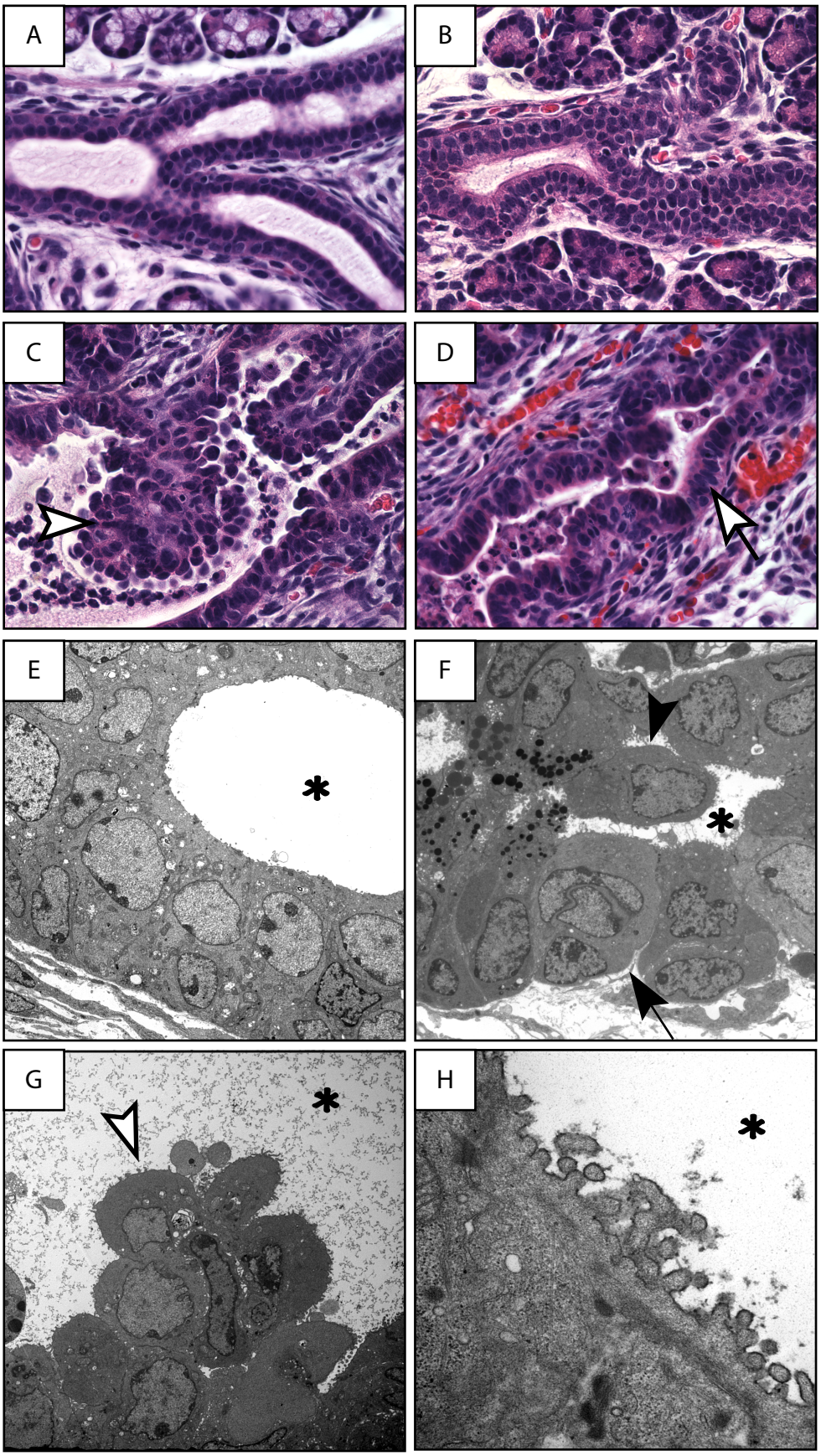

A
B
C
D
E
F
G
H

Figure 4. Ductal outgrowths, nuclear crowding, and cell-cell adhesion defects in mutant salivary glands. Wild type (A-B & E) or mutant (C-D & F-H) 1dpp salivary glands were analyzed by H&E staining (A-D) and at the ultrastructural level by EM (E-H). Wild type ducts showed well-ordered rounded nuclei in a bilaminate architecture. However, mutant ducts showed numerous ductal outgrowths (white arrow heads in C & G), nuclear crowding, and frequently lose bilaminate architecture (white arrow in D). Mutant ducts contained rounded cells in the ductal wall (black arrow in F) and shed cells in the lumina (black arrow head in F). Finally, mutant ducts also exhibited increased apical activity (H). Asterisks: lumen. A-D: 63x, E-G: 2,650x, H:, 25,000x.

mutant ducts showed ductal outgrowths with loosely adherent cells (Fig. 4C), and ductal nuclei were often elongated and crowded in to a single, pseudostratified layer (Fig. 4D). Ultrastructural examination of mutant ducts confirmed these observations and showed rounded cells within the ductal walls and areas of gross disorganization (Fig. 4F-G), as compared to wild type ducts (Fig. 4E), which have smooth baso-lateral and apical surfaces and cells that were completely adherent to one another. Lastly, the apical surface of the p120 negative ducts was covered with blebs, indicative of high apical activity (Fig. 4H), whereas wild type apical surfaces showed no such structures (data not shown).

p120 specifically regulates cadherin complex levels *in vivo*.

We previously demonstrated that p120 levels directly determine cadherin levels *in vitro* (Davis et al., 2003b; Ireton et al., 2002b). Therefore, salivary glands (Fig. 5A-C), lachrymal glands (Fig. 5D-F), epidermis, and hair follicles (Fig. 5G-I) were co-stained for p120 and E-cadherin, to determine the effects of p120 loss on cadherin levels *in vivo*. In each case, we observed a strict correlation between the levels of p120 and the levels of E-cadherin, confirming the need for p120 in maintaining normal E-cadherin levels *in vivo*. Since β-catenin and α-catenin levels are dependent on their interactions with cadherins (Nagafuchi et al., 1991), we stained tissue sections for β-catenin and α-catenin. As anticipated, their levels are also linked to p120 levels in a dose dependent manner; no nuclear β-catenin was observed (not shown). It is important to note that while E-cadherin levels were dramatically reduced in all cells showing p120 loss, complete loss of E-cadherin was only observed sporadically in the adult lachrymal gland and that E-cadherin levels were affected concomitant with p120 loss.

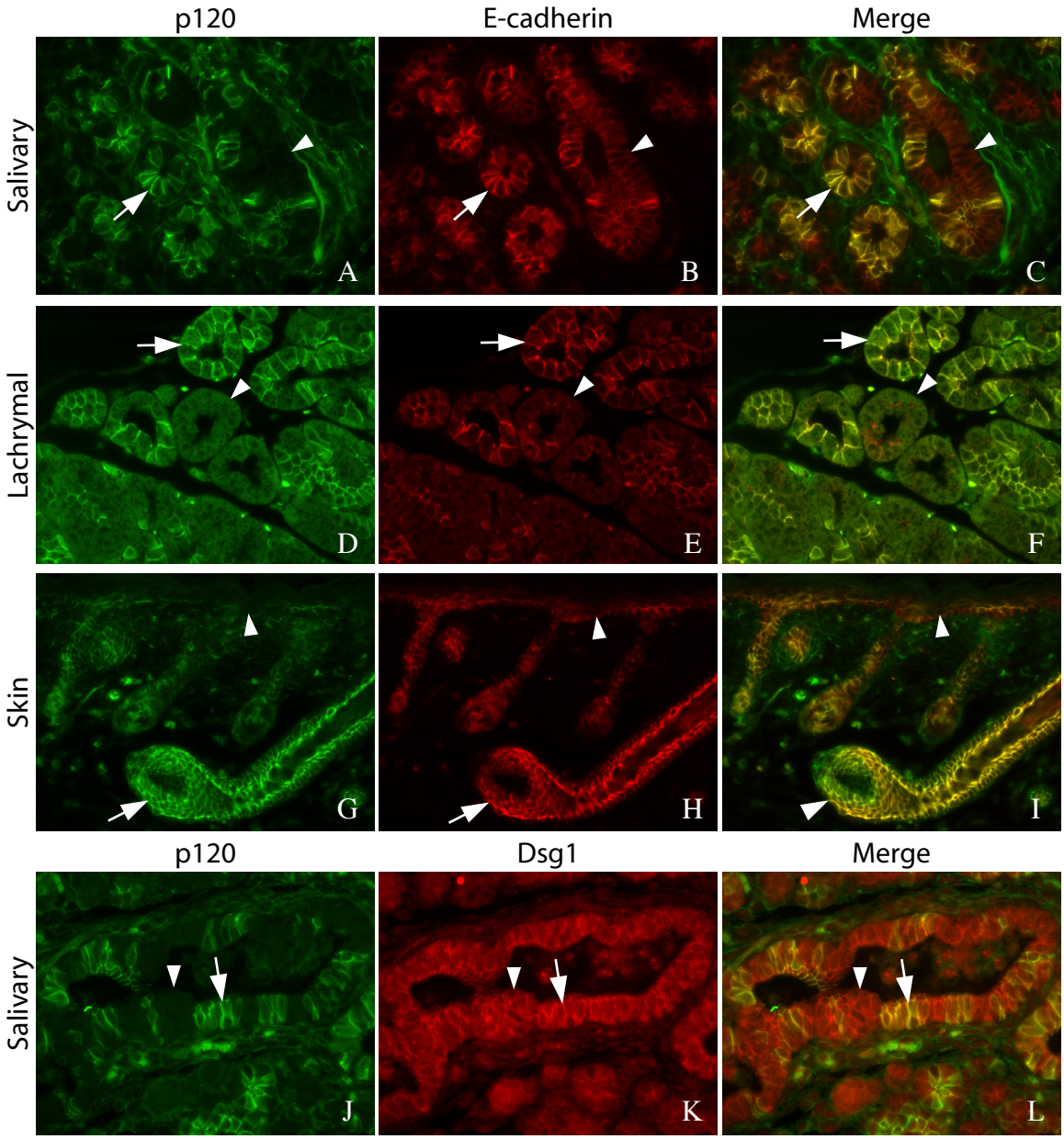


Figure 5. p120 specifically regulates cadherin complex levels *in vivo*.
Tissue sections with mosaic deletion of p120 were co-immunostained for p120 (A, D, & G) and E-cadherin (B, E, & H) or p120 (J) and Desmoglein 1 (K). Arrows point to cells with high levels of p120 and to the corresponding cells in the co-stain, while arrow heads point to cells with no p120 and to the corresponding cells in the co-stain. Note a direct correlation between p120 levels and E-cadherin levels and the lack of correlation between p120 and Desmoglein 1 levels.

The assembly of desmosomal and tight junctions requires the prior formation of adherens junctions (Pasdar and Nelson, 1988; Siliciano and Goodenough, 1988). Therefore, we stained sections for desmoglein 1 and occludin, members of desmosomal and tight junctions, respectively. Surprisingly, normal staining patterns were seen in non-hyperplastic ductal walls (Figure 5J-L), suggesting that desmoglein 1 and occludin were unaffected solely by the loss of p120. However, desmoglein 1 and occludin staining were perturbed in the ductal outgrowths (not shown). We attempted to confirm these data by western blot analysis of whole tissue lysates, but the high degree of mosaicism in the skin, the small size of the lachrymal glands, and drastic morphological abnormalities of the salivary glands in knockout animals made this impractical. However, ultrastructural examination by transmission electron microscopy of mutant salivary ducts surprisingly showed tight junctions, adherens junctions, and desmosomal junctions between most ductal cells (not shown).

These data are the first to demonstrate that E-cadherin levels are maintained *in vivo* by p120 expression. They also suggest that while cadherin levels were dramatically reduced in the absence of p120, sufficient levels of cadherins remain to establish adherens junctions. Furthermore, as p120 loss does not consistently affect other junctional complexes, the effects on the cadherin complex are specific to p120.

p120 ablation induces severe apoptosis and defects in cell polarity.

Lumen formation in the salivary gland reportedly occurs through apoptosis (Jaskoll and Melnick, 1999). Therefore we assayed apoptosis throughout salivary gland development by TUNEL staining (red staining in Fig. 6A-F). While apoptosis in p120-

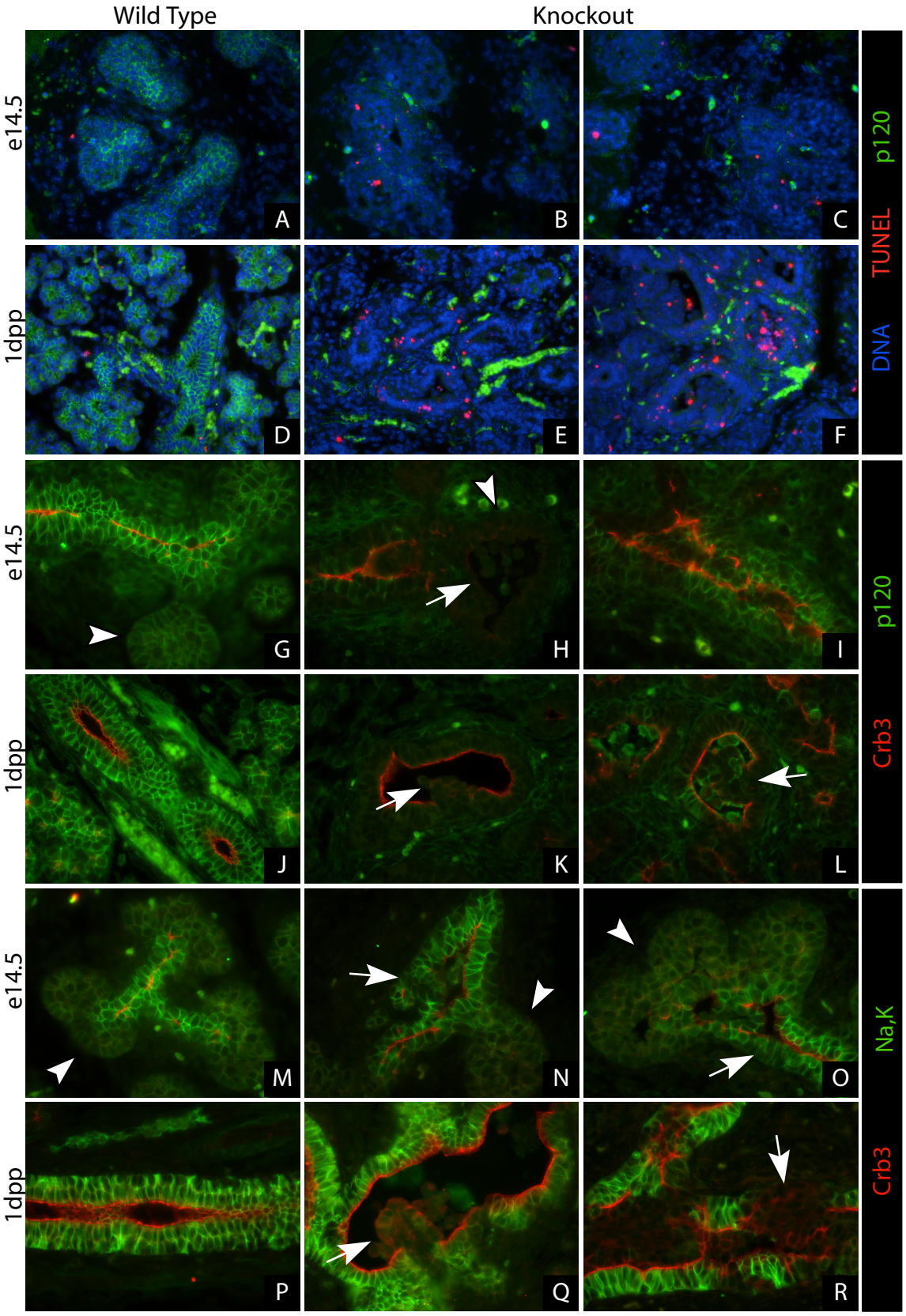

Wild Type
Knockout
e14.5
1dpp
e14.5
1dpp
e14.5
1dpp
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
DNA
TUNEL
p120
p120
Crb3
Na,K
Crb3

Figure 6. Increased apoptosis and loss of polarity in p120 knockouts
(A-F) e14.5 and 1dpp salivary glands were immunostained for p120 (green) and TUNEL (red). Knockout salivary glands (B-C & E-F) exhibit an increase in apoptosis beginning at e14.5 as compared to wild type glands (A & D). TUNEL staining did not localize to any particular structure, but was widespread. (G-L) Wild type and knockout, e14.5 and 1dpp salivary glands were examined for loss of polarity by immunostaining for p120 (green) and Crb3 (red) an epithelial-specific apical marker. Note the abnormal staining of Crb3 and areas that are missing Crb3 staining (white arrows). (M-R) To examine baso-lateral polarity, these animals were also stained for the baso-lateral marker Na^+, K^+ ATPase and co-stained with Crb3. The absence/mislocalization of Na^+, K^+ ATPase staining correlated strictly with that of Crb3 (white arrows). White arrowheads: end buds in e14.5 animals. While loss of polarity did not strictly correlate with p120 loss, it was widespread.

negative salivary glands was as much as ten-fold higher than in wild type salivary glands (28.5 ±4.6 vs. 2.8 ±0.8, respectively, p = 0.0001) it was not localized to the developing lumen. Rather, apoptotic bodies were observed throughout the epithelial tree, suggesting that p120 loss induced widespread apoptosis.

E-cadherin and adherens junctions are required to establish epithelial polarity (Faurschou and Borregaard, 2003). Furthermore, loss or mislocalization of ion transporters such as CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) and Na^+, K^+ ATPase are linked to pathological lumen dilation. Therefore, we examined the localization of the apical marker Crb3 and p120 (red and green, respectively; Fig. 6G-I) or Crb3 and the baso-lateral marker Na^+, K^+ ATPase (red and green staining respectively, Fig. 6M-R) in wild type and mutant salivary glands. Both Crb3 and Na^+, K^+ ATPase were expressed normally in many areas of mutant salivary ducts. However, both Crb3 and Na^+, K^+ ATPase were lost or mislocalized in ductal outgrowths of mutant glands (arrows, Fig. 6K-L & Q-R). Staining of both markers was also sporadically reduced or mislocalized in e14.5 mutant ducts (arrows, Fig. 6N&O). Finally, it is important to note that Crb3 and Na^+, K^+ ATPase were not expressed in dilated lumen of e14.5 end buds in either knockout (arrowheads, Fig. 6H & N-O) or wild type end buds (arrowheads, Fig. 6G & M).

These data support the hypothesis that lumen dilation may in fact be due to improper ion transport and the resultant secretion of water into the lumen rather than to apoptosis.

p120 loss causes severe neutrophil infiltration.

Nearly all knockout animals die soon after birth, however two knockout animals survived until adulthood. These were maintained in order to assess the potential effects of p120 loss on tumor formation. No tumors were observed in these mice, however by 6 to 8 weeks of age, pronounced hair loss was apparent. By 12 weeks of age, the hair loss was nearly complete and lesions covered large areas of the skin. At 16 weeks of age, the lesions were so severe it was necessary to sacrifice the animals. Upon histological examination of the epidermis of these mice, we observed severe inflammation and hyperproliferation of the epidermis and hair follicles. It is likely that hyperproliferation was a result of reactive changes caused by the inflammation, since epidermal thickening seemed to correlate with the degree of inflammation. However the small sample size makes this result inconclusive.

To address the potential correlation between p120 loss and inflammation, we examined neonatal and embryonic salivary glands for the presence of neutrophils (Fig. 7A-F), one of the earliest recruits in an inflammatory response (Faurschou and Borregaard, 2003). Surprisingly, embryonic mutants exhibited a neutrophil infiltration of the salivary glands as early as e16.5. Furthermore, while numerous neutrophils were observed in the stroma of mutant glands, we found dense neutrophil aggregates within the submandibular lumen of the larger ducts of 1dpp mutants (Fig. 7C). In fact, we observed neutrophils moving through presumptive lesions within the ductal walls of mutant glands (red stain, Fig. 7D-F). These lesions failed to stain for Crb3 (green stain, Fig. 7A-F) and had nuclei that were less compact than the surrounding epithelium (blue stain). Upon ultrastructural examination of mutant glands, we observed rounded nuclei within the

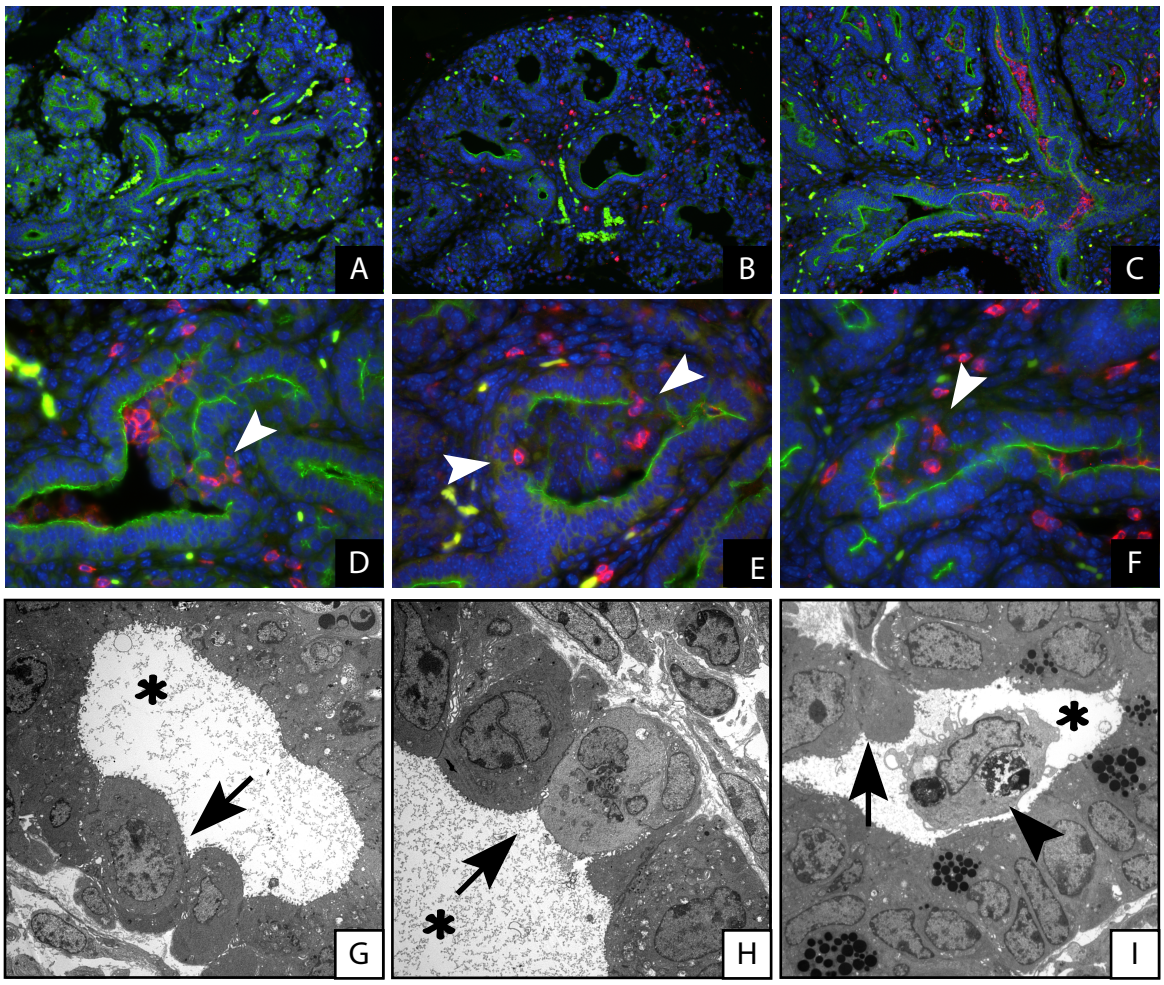


Figure 7. p120 mutants exhibit signs of inflammation.
(A-F) 1dpp salivary glands were stained for Crb3 (green), to delineate the lumen, and for neutrophils (red). Stromal staining of neutrophils was significantly increased in the mutant sublingual (B) and submandibular (C) glands, compared to wild type glands (A). Furthermore, there were dense neutrophil aggregates within mutant submandibular ducts (C). (D-F) higher magnification of mutant submandibular ducts shows neutrophils migrating across the ductal wall and into the lumen (white arrowheads). Note the loss of Crb3 staining where neutrophils are migrating across the epithelial wall. (G-I) Ultrastructural examination of mutant glands shows "lesions", rounded cells, in the ductal walls (arrows) and what is likely a luminal macrophage (arrowhead), filled with phagocytic vesicles. Asterisks: lumen. Yellow-green staining in (A-F) are autofluorescent red blood cells. (A-C) 20x, (D-F) 63x, (G-I) 2,650x.

ductal walls, which likely correspond to the lesions observed by immunofluorescence (Fig. 7G-H). Interestingly, we also observed cells that resemble macrophages within the lumina of mutant glands, suggestive of a more severe inflammatory response.

These data demonstrate that p120 loss results in neutrophil infiltration of the embryonic salivary gland, which is surprising given that prenatal animals are in the sterile environments of the uteri. Furthermore, the potential presence of macrophages in 1dpp salivary glands and the prolonged inflammation of the adult knockout skin suggest that p120 loss may lead to chronic inflammation.

Discussion

We have generated the first conditional knockout of p120 in mice, and our data conclusively demonstrate that p120 is directly and specifically required to stabilize E-cadherin in vivo. Consistent with a role for E-cadherin in development, p120 mutants exhibit a severe defect in ductal morphogenesis and acinar development. Surprisingly, we also demonstrated a neutrophil infiltration, and our data suggests that p120 loss may result in tissue damage and chronic inflammation. Finally, consistent with a role of p120 and E-cadherin in tumor progression, we observed widespread lesions bearing the hallmarks of neoplasias. While these data can not confirm a role for p120 in tumor progression, they go far to strengthen our hypothesis that p120 is a tumor modifier.

MMTV-Cre deletion of p120

Unexpectedly, MMTV-Cre deletion of p120 resulted in neonatal lethality. The conventional deletions of E-cadherin (Boussadia et al., 2002), Brca1 (Xu et al., 1999),

and Bclx(Wagner et al., 2000) are embryonically lethal, and MMTV-Cre-mediated conditional deletion of these genes successfully circumvents embryonically lethality. Thus it is surprising that MMTV-Cre deletion of p120 was lethal. The severe differences in phenotypes between MMTV-Cre deletion of E-cadherin (Wagner et al., 1997) and of p120 are likely due to strain variations, but it is interesting to note that the loss of no other member of the cadherin complex has as many down stream effects as the loss of p120. For instance, plakoglobin compensates for β-catenin loss in the cadherin complex, and defects observed in β-catenin null animals are attributed to defects in Wnt signaling (Huelsken et al., 2000). Although β-catenin connects the cadherin complex to the actin cytoskeleton, which is required for maturation of the adherens junction, β-catenin loss does not affect cadherin levels or α-catenin levels (Torres et al., 1997). Finally, E-cadherin loss in the skin upregulates P-cadherin levels (Tinkle et al., 2004). This is consistent with a role for p120 in establishing cadherin levels, where p120 acts as a fulcrum to set total cadherin levels. In contrast, siRNA-mediated p120 loss in A431 cells results in a simultaneous decrease in E-cadherin, P-cadherins, β-catenin, and α-catenin (Davis et al., 2003a). Therefore, it is possible that MMTV-Cre deletion of p120 induced a phenotype more severe than the deletion of E-cadherin, leading to postnatal lethality.

p120 and the cadherin complex

In vitro, p120 stabilizes cadherin levels (Davis et al., 2003b; Ireton et al., 2002b). To test the role of p120 in maintaining cadherin levels *in vivo*, we stained for E-cadherin, β-catenin, and α-catenin in three p120-negative tissues: salivary glands, lachrymal glands, and skin. Our data demonstrates that p120 is indeed required to stabilize E-

cadherin *in vivo*. Moreover, consistent with *in vitro* data, we observed a dose-dependent correlation between p120 levels and E-cadherin levels in tissues that are mosaic for p120 loss, whereas we typically saw no differences in staining patterns of Desmoglein-1 or Occludin, desmosomal junction or tight junction proteins, respectively. It is important to note that while cadherin levels were dramatically reduced, they were not eliminated. We next examined cell-cell junctions in wild type and mutant salivary glands by electron microscopy. Despite the loss of E-cadherin, we often observed tight junctions, desmosomal junctions, and well-formed adherens junctions. Although E-cadherin levels are dramatically reduced by immunostaining, these data indicate that enough cadherin complexes remain to generate adherens junctions and epithelial cell polarity. Since p120 family members (ARVCF and p0071) can compensate for p120 loss in the cadherin complex (Davis et al., 2003b), it is possible that they stabilized a small number of cadherin molecules. However, these proteins are not widely expressed and any compensation of p120 loss would likely be minimal in most tissues. Regardless, these data are the first to show that p120 loss *in vivo* leads to severe loss of E-cadherin, β-catenin, and α-catenin and suggest a primary role of p120 is to stabilize the cadherin complex. Furthermore, as p120 is the most ubiquitous of the p120 family members, its loss will likely result in E-cadherin loss in most epithelia.

p120 & Development

The conventional murine p120 knockout is embryonic lethal (personal communication, Walter Birchmeier, Berlin, Germany). To examine the roles of p120 in tumor progression, we generated a conditional knockout with the intention of studying

p120 loss in the mammary gland. Unexpectedly, p120 knockout animals die postpartum for reasons that are unclear. MMTV-Cre is expressed in multiple tissues, and we observed p120 loss in neonatal salivary glands, lachrymal glands, epidermis and hair follicles. While we noticed an inhibition of hair follicle development (data not shown), the MMTV-Cre deletion of p120 in the skin and hair follicles is extremely mosaic, making studies of p120 loss in the skin difficult. We observed near complete loss of p120 in 1dpp lachrymal glands and salivary glands, but the lachrymal glands were still in early stages of development. In contrast, wild type neonatal salivary glands had nearly completed morphogenesis by parturition, and p120-negative glands showed severe morphologic abnormalities. Therefore, we focused our efforts on understanding the effects of p120 loss in the salivary glands.

In knockout animals, p120 expression is depleted by e14.5, and the expression of E-cadherin is concurrently and drastically reduced. In addition, ductal lumina fill prematurely, large fluid-filled spaces are found within the end buds, and single cells are apparently suspended within the fluid of the dilated lumina. As apoptosis reportedly accounts for lumen formation in wild type glands (Jaskoll and Melnick, 1999), we stained embryonic and neonatal salivary glands for apoptosis by TUNEL. Apoptosis did not localize to the end buds or ductal lumen, despite being increased over wild type glands. Thus it is unlikely that increased apoptosis caused the premature lumen dilation. Loss of polarity and/or the mislocalization of ion channels such as CFTR and Na^+, K^+ ATPase cause pathologic lumen dilation in cystic fibrosis and polycystic kidney disease. As adherens junctions are required to establish polarity, we stained for Crb3, an epithelial apical marker, and Na^+, K^+ ATPase, a baso-lateral marker. Generally, e14.5 mutant

glands showed correct localization of these markers. However, we did observe sporadic loss/mislocalization of Crb3 and Na^+, K^+ ATPase along the ductal walls and a complete absence of these markers in luminal cells of the end buds. Based on these data, we propose the following model. p120 loss results in reduction in E-cadherin levels. In end buds, where cadherin levels are lower than in developed ducts, E-cadherin levels sporadically fall below a threshold necessary to achieve polarization. As these cells differentiate into ductal cells, they are unable to properly localize ion transporters like Na^+, K^+ ATPase and begin pumping ions into the ductal lumina and microlumina. As the ion concentration increases, water moves into the lumen to re-establish osmotic equilibrium. The water moves laterally, towards the distal and proximal ends of the ductal tree. As the intraductal pressure increases, loosely adherent cells within the end buds are forced to dissociate, resulting in large fluid-filled spaces.

In branched organs like the lung, ductal extension and branching is directed by a complex interplay of signals sent from the mesenchyme to the developing ductal tree and back. For instance, mesenchymal FGF-10 is a chemo-attractant that induces lung branch extension (Cardoso, 2001). As the branch lengthens, FGF-10 induces BMP-4 expression in the branch tip epithelium, which inhibits proliferation. As proliferation is inhibited at the distal tip, branching begins in adjacent epithelium. While the exact signals regulating each phase of salivary morphogenesis are unknown, it is clear that FGF's and BMP's are involved. It is interesting to note that growth factor receptors bind and are regulated by cadherin complexes (Qian et al., 2004). Also, despite the overall loss of β-catenin, E-cadherin loss may lead to an upregulation of β-catenin transactivation (Stockinger et al., 2001), which is responsible for upregulating BMP-4 in human colon cancer cells (Kim et

al., 2002). Thus, while E-cadherin may directly account for many of the observed phenotypes, it is likely that the highly controlled signaling cascades, which mediate ductal extension and branch morphogenesis, are affected by p120 loss and the disorganization of the end buds.

e17.5 and 1dpp knockout animals revealed a distinct lack of secretory acini, as compared to wild type animals. Interestingly, MMTV-Cre-mediated deletion of E-cadherin results in massive apoptosis of secretory alveoli in mutant mammary glands (Boussadia et al., 2002). Consistent with this, p120 loss resulted in an increase in apoptosis beginning at e14.5. By 1dpp, apoptosis in knockout animals was 10-fold higher than in wild type animals. Thus, it is conceivable that p120 loss induces apoptosis in newly formed acini or in acinar progenitor cells, thereby preventing acinar differentiation. A lack of acinar-specific markers prevents validating this hypothesis.

p120 & Inflammation

p120 loss resulted in a severe neutrophil infiltration of the salivary glands and a prolonged inflammatory response in the two knockouts that survived until adulthood. These data suggest p120 loss induced a chronic inflammatory response. Consistent with this, p120 loss occurs in nearly all cases of IBD (Karayiannakis et al., 1998). Moreover, expressing DN-N-cadherin in the colon, sequesters p120 thereby downregulating endogenous E-cadherin, and generates IBD-like symptoms (Hermiston and Gordon, 1995). Although gut microflora are necessary for IBD in mouse models (Jiang et al., 2004), p120 loss induced neutrophil infiltrates in the sterile environment of the placenta. Thus the ductal lesions may generate “wounded tissue” signals. Alternatively,

neutrophils help clear apoptotic cells in an acute response but not through phagocytosis. Therefore, the presence of phagocytic cells in the ductal lumina again suggests a more chronic response.

p120 & Cancer

In vivo, p120 loss and cadherin loss correlate in a wide range of cancer types (Thoreson and Reynolds, 2002). Furthermore expression of dominant negative N-cadherin, which sequesters p120 and induces loss of endogenous E-cadherin, generates adenomas and an IBD-like phenotype (Hermiston and Gordon, 1995). These data suggest that p120 plays a role in tumor progression, but no data directly demonstrates a role for p120 in this process. To determine the effects of p120 loss on tumor progression we attempted to target p120 loss to the mammary gland. Despite the untimely death of our p120 mutant animals we observe ductal lesions in the salivary glands that bear hallmarks of precancerous neoplasias.

In e14.5 knockout salivary glands, when p120 levels are first depleted, we observed ductal outgrowths. These masses grew throughout salivary gland morphogenesis, developing into hyperplasias and papillary structures that were clearly displacing the surrounding tissues by 1dpp. Many of these growths actively shed cells into the lumen of the salivary ducts, and exhibited a loss of cell-cell adhesion at the ultrastructural level. Although there was no significant difference in BrdU staining between wild type (4.5±0.8% BrdU positive ductal cells) and p120 knockout (4.8±0.9%) 1dpp epithelium (not shown), these growths did stain positively for BrdU, indicating that they were still growing. In the absence of survival cues or mutations in the apoptotic

machinery, unregulated growth should trigger apoptosis. However, apoptotic bodies did not concentrate within these growths, despite the fact that apoptosis rose 10-fold in knockout animals by 1dpp. Finally, polarity markers such as Crb3 and Na^+, K^+ ATPase were consistently lost or mislocalized in the ductal outgrowths. Thus, it is clear that these outgrowths, both hyperplasias and papillary structures, exhibited characteristics of precancerous growths: sustained abnormal growth, inhibition of apoptosis, and loss of polarity.

In addition to the hyperplasias and papillary structures in p120 negative ducts, we observed nuclear crowding, pseudostratification, and a loss of the bilaminate architecture that is typical of wild type salivary ducts. In fact, the sometimes rounded cells of the p120-negative salivary ducts (Fig. 4F & G) resemble SW48 adenocarcinoma cells grown in vitro. Like the p120-negative ducts, SW48 cells have reduced p120 levels and low levels of E-cadherin (Ireton et al., 2002a). These cells are fairly rounded and grow in linear arrays, with short stretches of E-cadherin-positive junctions connecting them—as if there are too few cadherin complexes to form circumferential adherens junctions. When p120 is re-expressed, cadherin levels rise, and cells are able to make circumferential cadherin-positive junctions. Therefore, it is interesting to hypothesize that pseudostratification, a hallmark of neoplasias, may be due to a loss of cadherin and the cell's inability to form circumferential adherens junctions.

In summary, our data are the first to conclusively demonstrate that p120 stabilizes E-cadherin levels in vivo. We show that p120 is also required for glandular morphogenesis: including ductal extension, proper branching, and acinar development. Furthermore,

although our data can not confirm a role for p120 in tumor progression, the loss of E-cadherin and the presence of lesions bearing hallmarks of precancerous growths strengthen our hypothesis that p120 is a tumor modulator.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

To directly determine the role of p120 in the E-cadherin complex, I have generated p120-specific siRNA for the *in vitro* knockdown of p120 and a floxed-p120 mouse for the *in vivo* ablation of p120. When these studies were initiated, the data implicating p120 as a regulator of E-cadherin function employed deletion mutants that abrogated binding of p120 to E-cadherin (Thoreson et al., 2000; Yap et al., 1998). However these data were inconclusive since multiple proteins bind the same region of E-cadherin as p120, Hakai and Presenilin-1 for instance (Baki et al., 2001; Fujita et al., 2002). Directly establishing a role for p120 in E-cadherin function requires expressing p120 mutants that are unable to bind E-cadherin or re-expressing p120 in p120-null cell lines. For reasons that are still unclear, overexpression of p120 is not tolerated. Therefore we are unable to overexpress p120 mutants that do not bind E-cadherin. Furthermore, despite extensive searching, there is a distinct lack of p120-null lines. By knocking down and deleting p120, my work has finally enabled us to directly examine the role of p120 in cadherin function.

siRNA-mediated knockdown of p120 *in vitro* resulted in the dramatic loss of the entire cadherin complex. Ireton et al. report a similar loss of E-cadherin in the p120-defecient SW48 colon adenocarcinoma cell line (Ireton et al., 2002b). However, this work could not generalize the need for p120 in maintaining E-cadherin levels, as this data is based on a single cancer cell line. Using siRNA to knockdown p120 in multiple cell

lines, I have shown that p120 levels determine E-cadherin levels in a dose-dependent manner. I also observed a concurrent loss of □-catenin and □-catenin, which are known to be stabilized by E-cadherin (Nagafuchi et al., 1991). These data demonstrate that p120 is indeed required to maintain normal levels of E-cadherin and members of the cadherin complex *in vitro*.

To test whether or not p120 is required to stabilize any cadherin to which it binds, I knocked down p120 in the A431 epithelial cell line, the HUAEC primary endothelial line, and the C2C12 myoblast line. In the A431 cells, I observed dramatic and simultaneous reduction in both E-cadherin and P-cadherin. I also observed a reduction of VE-cadherin in HUAEC's and a reduction of N-cadherin in the C2C12's. Simultaneous destabilization of E-cadherin and P-cadherin suggests that p120 controls total cadherin levels in a given cell, and destabilization of VE- and N-cadherin, in addition to E- and P-cadherin, suggests that p120 will stabilize any cadherin to which it binds.

I conditionally deleted p120 in mice to examine the role of p120 *in vivo*. The conventional knockout of p120 is embryonic lethal (personal communication, Walter Birchmeier, Berlin, Germany), therefore I intended to circumvent lethality by deleting p120 in the mammary gland using MMTV-Cre. MMTV-Cre is primarily expressed in the salivary glands and mammary glands, but it is also expressed in many other tissues (Wagner et al., 1997). Despite the "leaky" expression of MMTV-Cre, targeted deletion of E-cadherin (Boussadia et al., 2002), Brca1 (Xu et al., 1999), and Bclx(Wagner et al., 2000) successfully circumvents embryonic lethality associated with the conventional deletion of these proteins. Therefore, we were surprised when MMTV-Cre-mediated deletion of p120 killed most knockout animals within one or two days of birth. Air was

often found within the stomachs and intestines of neonatal knockout animals, suggesting an inability to maintain separation between the airways and the gastro-intestinal tract. This phenotype is indicative of a cleft palate, yet no clefting or p120 loss was observed in the palate. However, removing pups from the dam prevented suckling and prolonged their life (data not shown). These data suggest death was associated with feeding, but the exact cause of death is unknown.

p120 is required for proper epithelial morphogenesis. MMTV-Cre-mediated p120 loss occurred in the neonatal salivary glands, lachrymal glands, and skin, and in each instance, I observed developmental abnormalities. The defects in the salivary glands were most severe, likely due to the early deletion of p120 at e14.5. In salivary glands, I observed an immediate reduction of E-cadherin and dilation of the lumen. This was followed by abnormal ductal extension and branching, a lack of acini, and widespread ductal outgrowths. In neonatal lachrymal glands, I observed premature and excessive ductal dilation, but little else. At 1dpp, the lachrymal glands were still immature, and it is likely that more pronounced phenotypes would have been observed had the animals lived longer. In postpartum skin, p120 loss was extremely mosaic. Despite this, there were half as many well-developed hair follicles in knockout animals as compared to wild type animals. Likewise, the two knockout animals that survived until adulthood experienced widespread and persistent hair loss beginning at six weeks of age. Hair follicles progress through cycles of attrition and growth, suggesting that p120-negative hair follicles were unable to regenerate. Based on this, I suspect that loss of p120 in the salivary glands would have prevented salivary gland development altogether and that later deletion would have lessened the severity of the phenotype. Consistent with this, MMTV-Cre

deletion of E-cadherin occurred in the mammary gland towards the end of pregnancy and only resulted in the apoptosis of mammary alveoli at the onset of lactation (Boussadia et al., 2002), whereas the mammary ductal tree completely failed to form in each of two p120 knockouts that survived until adulthood. Together, these data indicate that p120 is critical for epithelial morphogenesis, and suggest that the timing of p120 loss may dictate the severity of the phenotypes observed.

To evaluate the role of p120 in stabilizing cadherin levels, I examined p120-null tissues from neonatal animals. At 1dpp, complete p120 loss was observed in the salivary glands and lachrymal glands, and mosaic p120 loss was observed in the epidermis and hair follicles. In each tissue, there was a dose-dependent correlation between p120 levels and E-cadherin levels. There was also a dose-dependents reduction in □-catenin and □-catenin levels. As MMTV-Cre targets epithelia, we were unable to assess the effect of p120 loss on other cadherins, such as VE-cadherin and N-cadherin. However it is clear that p120 indeed stabilizes the E-cadherin complex *in vivo*.

Desmosomes and tight junctions were largely unaffected by p120 loss. Assembly of desmosomal junctions and tight junctions requires the E-cadherin complex and the formation of adherens junctions. In turn, establishment of polarized epithelia requires these three junctional complexes. While E-cadherin levels were dramatically reduced in p120-null tissues, they were not completely eliminated. Furthermore, adherens junctions were visible by electron microscopy. Thus desmoglein-1 and occludin, desmosomal and tight junction proteins, respectively, were not generally affected by p120 loss. This demonstrates that the effects of p120 on the cadherin complex were specific to E-

cadherin and suggests that there are sufficient cadherin molecules to establish desmosomes, tight junctions, and cell polarity.

Interestingly, I observed sporadic yet widespread loss of cell polarity in p120-null salivary glands. Consistent with the establishment of desmosomes and tight junctions in much of the ductal epithelium, the apical polarity marker Crb3 and the baso-lateral marker Na^+, K^+ ATPase were properly localized. However these markers were frequently and concurrently mislocalized within ductal outgrowths. Moreover, localization of desmoglein-1 and occludin were also mislocalized in these outgrowths. These data suggest that polarity either disappears sporadically or occasionally never develops.

Our current data could not distinguish between loss of polarity or an inability to establish polarity. E-cadherin levels are lower and cell-cell adhesion is less established in the undifferentiated, developing end bud as compared to the differentiated duct (Hieda et al., 1996). Furthermore, end buds did not express Crb3 or Na^+, K^+ ATPase to the same degree as ductal epithelia and did not localize them in a polarized fashion. Therefore loss of p120 may occasionally force E-cadherin levels below a threshold that is necessary for the establishment of polarity. Thus, as p120-null epithelial cells of the end bud differentiate, they may fail to establish junctional complexes and polarity that is typical of ductal epithelium.

The osmotic force that drives fluid secretion and lumen filling is regulated by the flow of ions into the apical environment (Sullivan et al., 1998). Cell polarity is essential to establish an ion gradient, which is achieved by controlling the flow of Na^+, K^+, and Cl^-, and is regulated in part by Na^+, K^+ ATPase on the baso-lateral surface and by the CFTR Cl^- transporter on the apical surface. Thus, as the ionic strength of the lumen increases,

water flows into it (Figure 1). Moreover mislocalization of ion transporters, such as CFTR, is associated with pathological lumen dilation in diseases such as polycystic kidney disease and cystic fibrosis (Durie et al., 2004; Sullivan et al., 1998). e14.5 p120-null salivary glands exhibited regions with mislocalization of the apical marker Crb3 and the baso-lateral ion transporter Na^+, K^+ ATPase. It is likely that mislocalization of the ion transporters results in deregulation of the transepithelial ion gradient and premature lumen dilation (Figure 2). In support of this idea, CFTR null animals also exhibit increased lumen dilation of the salivary glands (Durie et al., 2004). Furthermore, CFTR mutants exhibit thickening of the luminal fluid (inspissation), and fibrillar material within the lumina. Interestingly, p120-null glands contain electron rich deposits within the lumina, and the increased mucin within ducts of sublingual glands suggests inspissation. Thus it is a strong possibility that premature lumen dilation observed in p120-null salivary glands is a result of mislocalized ion transporters.

Loss of end bud polarity does not explain the extensive disorganization and lumen dilation seen within these structures. I observed a distinct lack of Crb3 and Na^+, K^+ ATPase staining within the dilated lumen of p120-null end buds. However, salivary end buds do not contain lumen or localize Crb3 and Na^+, K^+ ATPase to the apical and baso-lateral membranes, respectively, and adhesion between cells of the end bud is weaker than that between cells of the duct (Hieda et al., 1996). It is likely, that intraductal pressure increases when water moves into the unpolarized ductal lumen to correct the osmotic disequilibrium. As a result, it is possible that weak cell-cell adhesions within the end bud are strained and are forcefully broken. Consistent with this idea, wild type end buds are bulbous in appearance and constrict as they meet the duct, whereas many of the

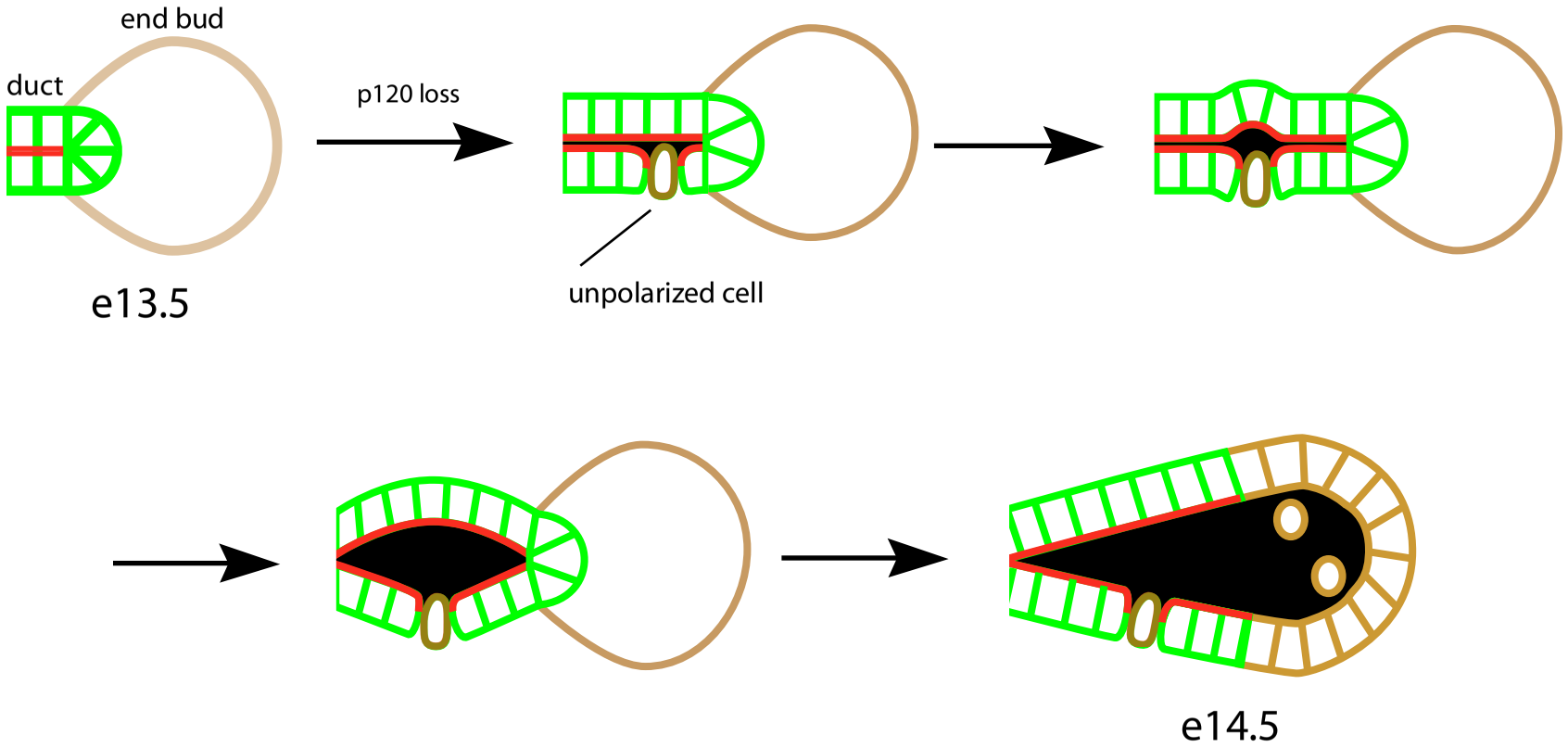


Figure 1. A morphogenetic model of premature lumen dilation.
p120 depletion in embryonic salivary glands occurred between e13.5 and e14.5. Concurrently, we observed loss of E-cadherin and sporadic loss of polarity. I speculate that loss of polarity results in increased luminal ionic strength and water secretion. As water flows into the lumen, the duct dilates. Increased intraductal pressure forcibly separates weakly adherent cells of the end bud.

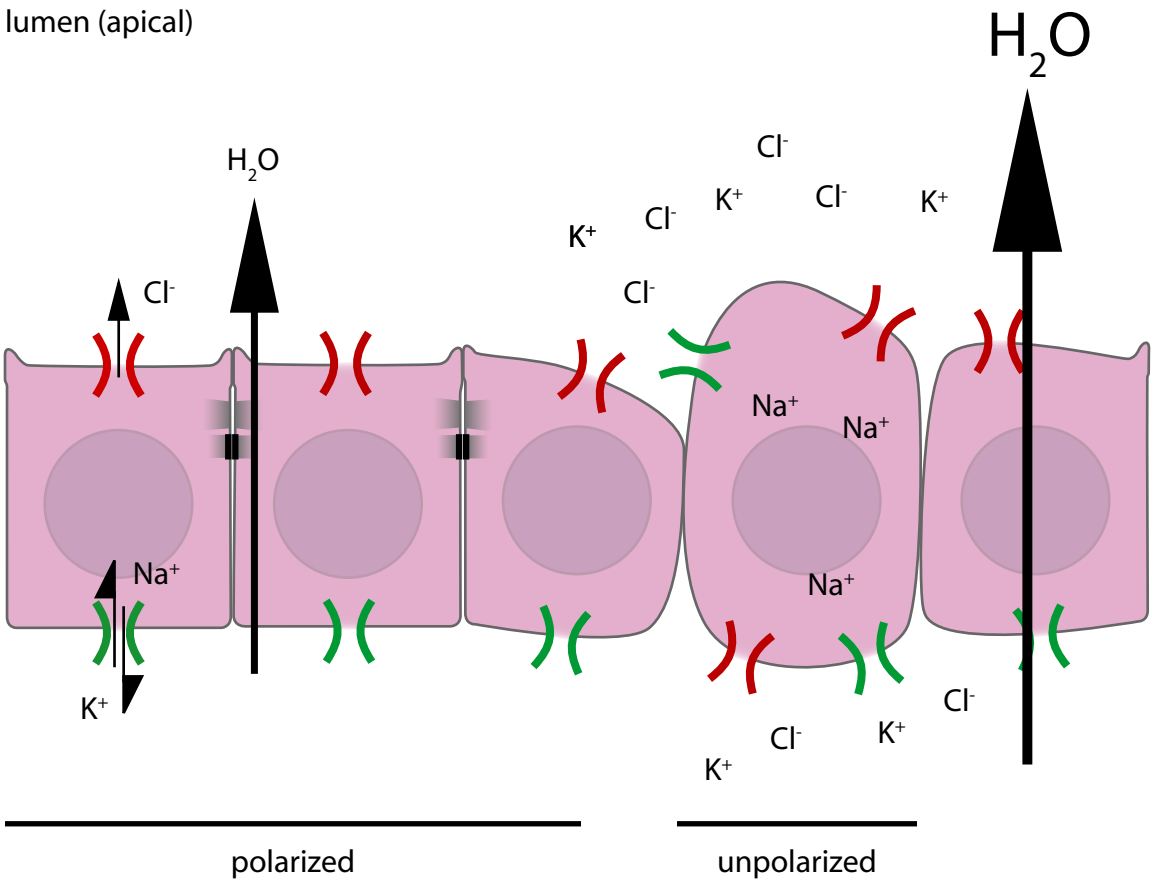


Figure 2. A mechanistic model of lumen dilation.
In epithelia, polarized localization of ion transporters enables the establishment of ion gradients that drive water secretion into the lumen. However, in unpolarized epithelia, ion gradients are enhanced by the mislocalization of ion transporters. As the luminal ionic strength increases, ductal cells upregulate water secretion to restore osmotic equilibria.

dilated end buds have lost the constriction between the end bud and the beginning of the ducts—as if the ducts and end buds of p120-null salivary glands have been inflated.

Lumen formation within the salivary gland reportedly occurs through apoptosis. Thus, apoptosis due to anoikis may explain the increased lumen size in e14.5 p120-null salivary glands, but my data are not consistent with such a mechanism. Concurrent with p120 loss, I observe E-cadherin downregulation in e14.5 salivary glands, and the number of apoptotic bodies also increases in these glands. However, apoptosis is widespread throughout the salivary epithelium and does not localize to p120-null end buds or lumen. Thus is seems unlikely lumen are forming in p120-null salivary glands due to apoptosis.

Loss of polarity may also explain the growth of the ductal masses in p120-null salivary glands. In polarized epithelia of the salivary glands, EGFR localizes to the baso-lateral membrane (Gresik et al., 1997; Kashimata et al., 2000). Moreover, EGF is secreted into the saliva where it contributes to gastro-intestinal epithelial turnover. Though tight junctions presumably prevent apical EGF from binding baso-lateral EGFR, ductal outgrowths in p120-null salivary are unpolarized. Therefore it is likely that EGFR is also mislocalized to the apical membrane where it may interact with luminal EGF to promote cell growth. A similar mechanism for aberrant cell growth occurs in autosomal dominant polycystic kidney disease (Du and Wilson, 1995). Immunolocalization of EGFR within the developing p120-null salivary glands could support this hypothesis, but confirmation requires a demonstration of EGFR activation. Such a finding would suggest that chronic proliferation in these lesions might have allowed the accumulation of mutations necessary to transition into frank cancer, if the animals had lived longer.

It is unclear why salivary acini did not develop in p120 knockout animals. The cues necessary for salivary terminal differentiation are not well characterized, but based on studies of lung, mammary gland, and pancreatic development, acinar differentiation is likely to require a complex interplay between the stroma and epithelium. The general disorganization observed in p120-null end buds may prevent signaling cues from being properly coordinated. As the end buds are also the site of ductal extension and branching, this idea is consistent with the fact that p120-null salivary glands branch and extend erratically. Alternatively acinar precursors may die by apoptosis. In fact, massive apoptosis is observed in p120-null salivary glands, and observation consistent with increased apoptosis resulting from MMTV-Cre-mediated deletion of E-cadherin, where mammary secretory alveoli undergo striking apoptosis and involution within a few days of E-cadherin loss (Boussadia et al., 2002). Thus apoptosis of acinar precursors may account for the failure to develop acini in p120-null salivary glands. Neither of these hypotheses is mutually exclusive, however we might exclude a role for apoptosis by growing excised salivary glands in vitro, pharmacologically preventing apoptosis, and analyzing acinar differentiation.

In conclusion, my work demonstrates that p120 has a critical role in stabilizing E-cadherin. Although I was unable to demonstrate a conclusive role for p120 in tumor progression, my results strengthen the hypothesis that p120 is a tumor modifier and may explain the concurrent loss of p120 and E-cadherin seen in a wide variety of tumor types. The next steps are already being taken by current lab members and are designed to reduce the risk of mortality due to p120 loss so we may finally determine the role of p120 in tumor progression.

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