AD

Award Number: DAMD17-96-1-6089

TITLE: The Role of KIP2 in Breast Cancer

PRINCIPAL INVESTIGATOR: Stephen J. Elledge, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: September 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010716 083

REPORT DOCUMENTATION PAGE		Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Annual (1 Sept 99 - 31 Aug. 00)

4. TITLE AND SUBTITLE

The Role of KIP2 in Breast Cancer

5. FUNDING NUMBERS

6. AUTHOR(S)

Stephen J. Elledge, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, Texas 77030-3498
E-Mail selledge@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The cell cycle is regulated by the action of a family of cyclin dependent kinases (Cdks) which catalyze particular cell cycle transitions. Cdks are positively regulated through interaction with cyclins and are negatively regulated through phosphorylation and through association with inhibitory proteins of the CIP/KIP and INK4 families. Our research has focused on the role of p57KIP2 in development and cancer. We have found that p57KIP2 is expressed in a highly cell type specific manner during embryonic development and in adult tissues being most highly expressed in terminally differentiated cells. Through analysis of p57KIP2 deficient mice, however, we have found that p57KIP2 is required for normal development of several tissues including the kidney, lens, muscle, and bone. We also discovered the p57 is involved in the human overgrowth and cancer predisposition disease Beckwith-Weidemann Syndrome, BWS. Current studies are aimed at understanding in greater detail the function of p57KIP2 and its possible role in human cancers.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	28
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Stephen J. Elledge, Ph.D. 10/19/00
PI - Signature Date

Table of Contents

Page 1	Front Cover
Page 2	SF 298
Page 3	Foreword
Page 4	Table of Contents
Page 5	Introduction
Page 9	Body
Page 13	Conclusion
Page 16	References
Page 24	Appendix
Page 24	Table 1
Page 25	Table 2
Page 26	Table 3
Page 27	Table 4
Page 28	Personnel and Publications

Introduction

The eukaryotic cell cycle is regulated primarily at two points, in G1 prior to entry into S-phase and in G2 prior to entry into mitosis. The commitment to a round of cell division is made at a point in G1, referred to as the restriction point in mammalian cells[1-4] or START in yeast[5]. Passage through the restriction point depends critically on mitogen signals, but once this point is passed, cells are committed to S-phase and the remainder of the cycle in a mitogen independent manner[4]. Passage through the restriction point is thought to be the primary event controlling cell proliferation. Therefore, elucidating how positively and negatively acting genes function to regulate the G1/S transition and how mutations in these genes disrupt normal cell cycle control has been a primary focus of cancer research. Central to this focus has been the investigation of the role of cyclin-dependent kinases (Cdk) in the control of cell proliferation.

Cyclins, Cdks, and positive growth control. Cdks are protein kinases that require association with cyclins and phosphorylation for activity[5-8]. Cyclins promote cell cycle transitions via their ability to associate with and activate their cognate Cdks[5-12]. Cyclins D and E function in G1[6, 10, 13-16], and overexpression of cyclin D1 or cyclin E shortens G1 and accelerates entry into S-phase[1, 3, 17, 18]. Amplification of cyclins, D1, D2 and E have been identified in several tumors[19-23]. Cyclin D1 was identified as the PRAD1 oncogene [24]. Cyclin A was identified as the site of integration of HBV in a hepatocellular carcinoma[25]. Taken together, these observations suggest that inappropriate activation of Cdks is a mechanism that cells frequently use to reach the oncogenic state.

Cyclins D1,D2, and D3 bind Cdk4 and Cdk6 kinases and can phosphorylate and inactivate Rb[6, 26-29]. Because D-type cyclins are required for proliferation only if cells have an intact Rb gene, it is thought that Rb inactivation is their primary role. Cyclin E binds to and activates Cdk2 and considerable evidence has accumulated indicating that cyclin E/Cdk2 is the primary kinase involved in the G1/S transition[14, 15, 30-33]. In addition, a close homolog of Cdk2 - Cdk3 - is also thought to play a unique role in the G1/S transition[31]. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition [34-36], while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry.

Although Cdks are thought to be the critical regulators of cell proliferation, little is known about how cyclin/Cdk complexes regulate cell proliferation during development. In this regard, we have performed an analysis of the expression of the major cyclins during mouse embryonic development and in adult tissues. We have discovered that general cyclins E, A, B, and F are expressed in all proliferating tissues while the D cyclins are distributed in a pattern distinct for each cyclin but which is a subset of the general cyclins (Parker, Harper and Elledge, unpublished results). This is consistent with the notion that D cyclins are the primary initiators of cell cycle entry and orchestrate development. We have recently observed that cyclin D1 is the only D-type cyclin induced when breast cells proliferate during pregnancy. We and our collaborators in the Weinberg laboratory discovered that development of the breast during pregnancy is dependent upon cyclin D1[37]. As mentioned above, amplification of D-type cyclins is frequently observed in breast cancer. This provides a link between development and cancer and indicates that the developmental history of the breast is relevant to its susceptibility to tumorigenisis.

Since the controls utilized during development to regulate cell proliferation are similar to those utilized in maintenance of the non-proliferative state in differentiated tissues, it is likely that these controls are reactivated or overcome in cancer. Another example of this comes from our observation that cyclin D1 is expressed at extremely high levels in the retina and is required for its development [37]. Presumably the inability to properly develop the retina in cyclin D1 mutants reflects an inability to overcome Rb. In what is clearly more than a coincidence, the retina is the same tissue in which high frequency tumors arise in Rb mutant humans. It is therefore likely that the Rb protein is important in both development of that tissue and its maintenance in the non-proliferative state. Our understanding

of the links between development and cancer is in its infancy and is an area in which there is a great need to increase our knowledge base.

Tumor suppressor proteins and negative growth control. Rb and p53[29, 38] are the most well understood tumor suppressors. Mutations in these are found frequently in many human cancers[39,40], and reintroduction of wild-type genes into p53$^-$ or Rb$^-$ tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis[28, 41, 42].

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors[39] including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included[43]. p53 deficient mice are prone to the spontaneous development of a variety of tumor types[44]. Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53[45-53]. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification[54, 55]. p53 regulates the expression of p21^{CIP1}, an inhibitor of G1-cyclin/Cdks, in response to DNA damage[56-60]. Using a p21 knockout mouse, we have determined that p21 is required for full function of the G1 checkpoint in response to γ-irradiation, although there is residual checkpoint function[56]. Furthermore, these mice do not show the high rate of spontaneous tumor formation seen in p53-deficient mice. It is not clear whether p53's role in oncogenesis is through its checkpoint or apoptotic deficiencies, or a combination of these.

The current view of the role of Rb in the cell ccyle is that hypo-phosphorylated Rb functions during G1 in part to block the activity of E2F and related transcription factors that are required for the expression of genes involved in S-phase[29]. Hyper-phosphorylation of Rb or association with DNA tumor virus oncoproteins such as E1A results in release of E2F and is correlated with passage into S-phase.

The above observations are consistent with a model in which increased cyclin/Cdk activity in tumors, whether by increased cyclin expression or decreased negative regulation, can overcome the cell cycle repression function of Rb via direct phosphorylation and inactivation of its growth inhibitory function. Rb therefore acts as a potential energy barrier in the pathway that cyclin/Cdks must overcome to activate cell cycle entry. Removal of the barrier (Rb) may reduce the levels of kinase activity required, but some Cdk kinase activity is still required for the process of DNA replication and can therefore act as a target of further negative regulation. In this model, p53 acts to reduce the frequency of mutations that lead to altered growth control and to kill cells that have undergone extensive damage or are inappropriately growing. To fully understand this aspect of cancer, cell cycle dysfunction, it is imperative that we have a complete understanding of the regulation of cyclin dependent kinases and their regulators in the tissues of interest.

The cell cycle and development: potential roles for Cdk inhibitors. Once proliferation and morphogenesis have constructed a particular structure, it is of paramount importance that the proliferative state cease and be replaced with a homeostatic state. While much attention has been focused on how cells enter the cell cycle, little is known concerning the strategies organisms employ to exit the cycle and maintain the non-proliferative state. This state is of great importance to an organism because the vast majority of its cells exist in a non-proliferative state throughout adult life. The inability to appropriately halt growth can lead to malformation during development, and to cancer. Thus, equally important in the execution of developmental programs is the arrest of growth once the program is complete. While the control of terminal differentiation promises to be complex, cell cycle arrest via inactivation of Cdks is likely to be a central feature. Recently a new class of Cdk regulatory molecules have emerged that are potential mediators of cell cycle exit and maintenance of the non-proliferative state. These are the inhibitors of cyclin-dependent kinases, CKIs. Currently two

structurally defined classes of CKIs exist in mammals that are exemplified by p21^{CIP1} [57-60] and p16$^{INK4/MTS1}$ [61-65].

Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control. Cdk inhibitory proteins are a group of proteins that associate with and inhibit Cdks. These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life. At the time of submission of this grant in December 1993, the first mammalian Cdk inhibitors p21$^{CIP1/WAF1}$ [57-60] and p16^{INK4a} [61] had only recently been identified. Subsequently, we and others identified additional inhibitors including p27, p57, p15, p18, and p19 (refs 61-69). We identified p21^{CIP1} in a two-hybrid screen designed to identify proteins that associate with Cdk2 [57]. Importantly, this protein was simultaneously cloned by several other laboratories. p21 was cloned as a p53 activated gene by the Vogelstein laboratory [59], as a Cdk associated protein by the Beach laboratory [58], and as an S-phase inhibitory cDNA in senescent cells [60]. Since then we and others have identified two other members of the p21 family, p27 and p57. p57, also known as KIP2 has been the focus of this study. It is expressed in the breast and is localized to 11p15.5m a locus involved in breast cancer (see below).

Involvement of 11p15.5, the location of KIP2, in human cancers including cancer of the breast. *Several chromosomal regions show frequent loss of heterozygosity(LOH) in breast tumors including but not exclusively 3p, 7q31, 11p15, 11q13 and 17p[reviewed in 96]. The chromosomal location of KIP2, 11p15.5, marks it as a candidate tumor suppressor gene of the breast. The involvement of 11p15 in the breast is well documented[113-119]. 35% of breast tumors show LOH at 11p15.5[119] and this LOH is associated with poor prognosis[119]. Furthermore, 11p15 LOH has been associated with metastasis[116] and there is evidence that 2 distinct breast tumor suppressor genes may reside at this locus[118].* 11p15 has also been intensively investigated because of frequent LOH at this locus in a number of other human cancers including bladder, lung, ovarian, kidney, and testicular carcinomas [reviewed in 70]. Several childhood tumors including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma show specific loss of maternal 11p15 alleles, suggesting a role for genomic imprinting. Chromosome transfer experiments have also indicated a tumor suppressor gene resides at this locus, the WT2 gene involved in Wilms' tumor and possibly rhabdomyosarcoma [reviewed in 71], either of which could be due to loss of a Cdk inhibitor. In addition, rearrangements in the 11p15 region are found in Beckwith-Wiedemann Syndrome (BWS) which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, visceromegely (enlarged organs) and an increased risk (7.5%) of childhood tumors [72,73]. Genetic analysis indicates maternal carriers, also suggesting a role for genomic imprinting[reviewed in 74]. Several features of KIP2 make it a reasonable candidate as a mediator of some phenotypes of BWS. First, a Cdk inhibitor could explain both overgrowth and tumorigenesis phenotypes. Furthermore, the expression pattern of KIP2 in mouse correlates with areas known to be affected in BWS including the tongue, kidney, muscle, and the eye. Third, KIP2 is imprinted and maternally expressed. Furthermore, LOH at 11p15 in Wilms' tumors are exclusively maternal, offering further support for the possibility that KIP2 might be the WT2 gene. LOH of the breast has not yet been examined for parental specificity of LOH. However, the potential for the existence of two tightly linked tumor suppressors[75] affecting the breast at 11p15 might complicate the analysis of parentally biased LOH depending on the relative frequency of the two events. Nevertheless, the biochemical properties of KIP2, its physical location and expression patterns suggest that it may be the tumor suppressor at 11p15.

A role for p57 in Beckwith-Wiedemann Syndrome.

As mentioned above, Beckwith-Wiedemann Syndrome (BWS) is a clinically variable disorder characterized by somatic overgrowth, macroglossia, abdominal wall defects and visceromegaly (83). Children with BWS are also susceptible to a variety of childhood tumors, including Wilms' tumor, hepatocellular carcinoma and rhabdomyosarcomas. The disease, which affects 1 out of every 13,700 live births each year, is genetically heterogeneous, with the majority of cases occurring sporadically. Familial cases, which represent ~15% of BWS patients, have helped establish a genetic linkage of BWS to human 11p15.5, where there is a large cluster of imprinted genes (84, 85). That defects in imprinted genes might explain the etiology of the syndrome was first suggested from observations that both familial and sporadic cases can be associated with 11p15.5 partial paternal uniparental disomies (UPDs) and trisomies with paternal duplications (86, 87).

Many of the key defects found in BWS patients could be explained by alterations in the control of cell proliferation, either in the context of organogenesis or tumorigenesis. Thus, candidate genes should be tied in some respect to the control of cell proliferation as well as being imprinted. Two candidate imprinted genes with these properties map to 11p15.5, *IGF2* and $p57^{KIP2}$. These genes have strikingly similar patterns of expression during development in mice and are expressed in all of the tissues affected by BWS (88, 89, 90).

IGF2 encodes a fetal-specific growth factor that is paternally expressed in both mice and humans (91, 92, 93). When the expression of *IGF2* was examined in BWS patients, it was shown to be elevated as the result of deregulation of its imprinting in some, but not all cases with normal karyotypes (94, 95). Furthermore, paternal trisomies and UPDs would be expected to double the expression of *IGF2* and could potentially account for overgrowth observed in these cases of BWS.

Maternally inherited loss-of-function mutations in the $p57^{KIP2}$ gene have also been identified in ~5-15% BWS cases examined (96, 97) and 30%-50% of familial cases cases (98). $p57^{KIP2}$encodes a member of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs), and is maternally expressed in all mammals examined to date (99,89,90,100,101). CKIs of this class inhibit G1/S phase cyclins, and the absence of $p57^{KIP2}$ has been shown to affect the ability of cells to exit from the cell cycle. The gene lies ~800 kb from *IGF2* in both mouse and human (102, 103, 104).

A small number of patients (<1%) have been identified with balanced translocations or inversions 3' of $p57^{KIP2}$ in a neighboring imprinted gene, *KvLQT1* (105, 106, 107). This gene encodes a voltage-gated potassium channel that is maternally expressed in humans in all tissues except the heart, but mutations in the gene have been implicated only in the cardiac arhythmia long QT syndrome, not BWS (108, 102). It has been suggested that the regulation of other genes in the locus, particularly $p57^{KIP2}$and *IGF2*, may be disrupted by the translocations. Indeed, in one such family, *IGF2* expression has been shown to be biallelic (109). Finally, the methylation imprint and expression of a recently described paternally expressed transcript within an intron of *KvLQT1, LIT1*, has been shown to be disrupted in over 50% of BWS patients examined (110, 111, 112). These groups suggest that this transcript may mediate the imprinting of other genes in the locus, although they disagree on the likeliest candidates (110, 111, 112). Several mouse models that shed light on the etiology of BWS have been generated. These studies have confirmed that embryonic growth in mice is very sensitive to the levels of the growth factor IGFII. When a mutated copy of the *Igf2* gene was inherited paternally, the offspring were 60% the size of their wild-type littermates (91). In contrast, in mice in which the cis-acting sequences that control *Igf2* imprinting were deleted (*H19_13*), *Igf2* was expressed from both parental chromosomes, and the offspring displayed somatic overgrowth and placentomegaly, but none of the other symptoms of BWS (113, 114). This mutation raised the tissue levels of IGFII 2-fold, but had a less pronounced effect on its circulating levels.

Mutations in the type 2 *Igf2* receptor gene (*Igf2r*), whose product binds IGFII and targets it for lysosomal degradation also caused elevation of IGFII (115, 116, 117). Circulating IGFII levels were elevated ~4-fold, and embryos died late in gestation with many, but not all, of the phenotypes of BWS, such as somatic overgrowth, placentomegaly, heart hypertrophy, omphalocele and adrenal cysts. In *Igf2r* and *H19_13* double mutants (114), IGFII levels were increased 7-11 fold, and the severity of

the phenotypes were more pronounced than in either single mutant. In these mice, however, the macroglossia, renal dysplasia and adrenal cytomegaly commonly found in BWS patients were missing. Overexpression of IGFII has also been achieved in mice carrying *Igf2* transgenes (118). These animals exhibited overgrowth, polydactyly and polyhydramnious, all symptoms of BWS patients. Together, these animal models support the hypothesis that BWS results from elevated expression of *IGF2*.

The impact of loss-of-function mutations in $p57^{Kip2}$ has also been examined in mice (119, 120). These mice show abdominal wall defects reminiscent of those seen in *Igf2r/H19* double mutants. They also display a unique set of defects such as renal dysplasia and adrenal cytomegaly, defects seen in BWS patients but not observed in mice with elevated IGFII expression. The somatic overgrowth commonly associated with BWS, however, was not observed. In addition, phenotypes not previously associated with BWS, such as lens and gastrointestinal tract abnormalities as well as skeletal defects were present in $p57^{Kip2}$-null mice.

These mouse models have failed to provide a good explanation for the fact that BWS patients with loss of imprinting of *IGF2* and those with mutations in $p57^{KIP2}$ are phenotypically indistinguishable. Zhang et al. (120) suggested that IGFII and p57 may act in opposing manners to control cell proliferation during development of human fetuses; that is, that a gain of function of *IGF2* may act similarly to a loss of function of $p57^{KIP2}$. We reasoned that if IGFII and p57 act antagonistically during development, a double mutant in which both BWS-potentiating mutations are present might exhibit phenotypes that are more severe than the sum of those in the single mutants. Such a mouse strain would mimic patients with UPD of 11p15.5, with respect to *IGF2* and $p57^{KIP2}$. To generate such a mouse we bred a loss-of-function $p57^{Kip2}$ mutation to an *Igf2* loss-of-imprinting mutation (*H19_13*) and screened for meiotic recombination between these tightly linked genes in the next generation. The double mutant mice exhibit aspects of BWS that have not been observed in other mouse models, such as macroglossia. In addition, they show an exacerbation of the placental and kidney dysplasias caused by the $p57^{Kip2}$ mutation alone. Significantly, we observed that a null mutation in *Igf2* can overcompensate for these severe placental and kidney dysplasias, leading us to suggest that the two genes act in an antagonistic manner in some tissues in the mouse.

The goals of our work were: 1) to determine whether p57 is imprinted in the breast, 2) to construct mice lacking p57, 3) to analyze the phenotype of mice lacking p57, 4) to analyze the role of the QT domain in p57 function by looking for binding proteins, and 5) to characterize the regulation of p57 and 6) to look for additional CKIs in the breast. To date, we have made significant progress on these initial goals. Our progress in these areas is summarized below.

Body

Aim 1: Determination of p57 imprinting status in the breast.

We have completed this Aim and described this in last years report..

Aim 2: Construction of mice deficient in KIP2. We completed this aim and discussed it in last years report.

Aim 3: Analysis of p57 mutants animals.

We have completed an exhaustive analysis of the mutant phenotypes present in the p57 mutant and p57/27 double and p57/p21 mutant animals in last two years and discovered multiple roles for these proteins in eye, lung, and muscle development. In the past year we have investigated the possible overlap between p57 loss and increases IGF2 growth factor expression in mouse models for the cancer syndrome BWS by making mice mutant for both p57 and H19, a negative regulator of IGF2 imprinting. H19 mutations express 2-fold more IGF2 and IGF2 overexpression has been implicated in BWS. The phenotypes of mice lacking these two genes are discussed below in the order in which we detected them.

A. Generating a p57^{Kip2}/H19 double mutant

The phenotypic consequences of mutations in *p57^{Kip2}* and *H19* are manifested only when the genes are inherited from mothers. Because these genes lie ~900 kb apart in the mouse, a double mutant strain could be generated by meiotic recombination between the existing mutations. Towards that end, we crossed heterozygous *p57$^{Kip2+/-}$* ; *H19*Δ13$^{-/+}$ males to C57BL/6 females and screened progeny for animals that were heterozygous for both *p57^{Kip2}* and *H19*Δ13 (*p57H19*). Among 481 offspring, we identified two *p57H19* recombinants, a frequency consistent with the estimate of the genetic distance between the genes (0.5 cM). One recombinant, a male, was fully viable and fertile and was used to establish the line. The other recombinant, a female, failed to give birth to viable *p57H19* mice.

Loss of *Igf2* imprinting, and the absence of maternal *p57^{Kip2}* and *H19* expression were confirmed by RNAse protection and RT-PCR assays. The imprinting and expression of *Kvlqt1* was unchanged (data not shown).

B. Perinatal Lethality

Maternal inheritance of a null allele of *p57^{Kip2}* is lethal, with 10% of offspring dying *in utero* and the remainder dying within the first two weeks after birth (120). We were unable to recover live *p57H19* mice at birth, suggesting that the double mutant phenotype is more severe. Furthermore parturition invariably occurred at 19 d.p.c., at least one day earlier than in *p57^{Kip2}* single mutants. At 18.5 d.p.c., *p57H19* embryos were present at approximately the expected frequency (Table 1), suggesting that death occurred during or shortly after delivery.

C, Prenatal Growth

One of the characteristics of BWS is prenatal somatic overgrowth. It had been shown previously that offspring inheriting the H19_13 mutation maternally are born 30% bigger than their wild-type littermates (113, 114). *p57^{Kip2}* mutants, on the other hand, display no somatic overgrowth at birth (119, 120). Although *p57H19* embryos were indistinguishable in weight from wild-type littermates at e18.5, they were ~20% larger at e16.5-17.5 just as was seen in the single *H19Δ13* deletion at the same time (113). It is conceivable that the gain in growth rate mediated by the loss of *Igf2* imprinting is compromised later in gestation by *p57*^{Kip2}-induced defects.

BWS patients often display specific organomegaly, most often affecting the tongue and adrenal glands, and less frequently the liver, kidney and heart. To distinguish specific organ overgrowth from generalized overgrowth in *p57H19* mutant mice, we calculated organ weights at 18.5 d.p.c. as a percentage of total body mass. By this criterion, neither *H19_13* nor *p57*^{Kip2} mutant mice showed specific organ overgrowth (data not shown). In *p57H19* offspring the only organ that displayed significant overgrowth was the tongue which was 122% of wildtype ($p<0.001$) (Table 2). If we tabulate those animals whose normalized growth was one standard deviation above the mean of their wild-type littermates, overgrowth of the kidney, heart, as well as overall somatic overgrowth, are observed more frequently in *p57H19* mutants than in wild-type littermates (Table 3).

D. Placental defects

The most dramatic overgrowth phenotype observed in *p57H19* embryos was placentomegaly, with 18.5 d.p.c. placentas weighing on average 190% of those of wild-type littermates. On histological analysis, mutant placentas were highly disorganized in the labyrinthine layer, where both *p57*^{Kip2} and *Igf2* are expressed. Mutant placentas also displayed fibrin cysts, apoptotic cells and large accumulations of red blood cells. A similar disorganization was seen at 16.5. and 17.5 d.p.c., suggesting that this effect occurs before the degeneration of the organ that normally occurs in late gestation. It is unclear whether red blood cells accumulate because of the tubule networks through which maternal and fetal blood flow are not established, are breaking down through cell death, or are blocked by surrounding cellular overgrowth. *p57*^{Kip2} single mutant animals showed related morphological placental defects, including reduced vascularization of the labyrinthine zone caused by

an overproliferation of trophoblast cells. In addition, the occurrence of hyaline membranes, a response to endothelium damage, was observed and was thought to result in a blockage of the blood supply (121). The *p57H19* phenotype that we observed, however, was far more severe.

In *p57H19* mutants there was a direct correlation between the placental weights and disruptions in placental cellular architecture, suggesting that the observed placentomegaly is caused primarily by the increase in red blood cell volume. Interestingly, there was actually a modest positive correlation (r=0.37) between the disorganization of the placenta, as reflected by its weight, and the size of the embryo. Thus the placental dysmorphologies did not compromise fetal growth.

The placental morphology of BWS patients has been examined in a few cases where disease was anticipated prenatally or following stillbirths or neonatal death. In these cases, placentas contained large cysts in stem villi. Some of the terminal villi in individual cases were filled with blood, and the trophoblast layer was hyperplastic in some, but not all, cases (122). Despite the significantly different architectures of the human and mouse placentas, these phenotypes are similar to those we observe in the *p57H19* mice.

E. Kidney Dysplasia

Kidneys in *p57H19* mice were underdeveloped in the medullary region, where $p57^{Kip2}$ is normally expressed. This region normally consists of stromal mesenchymal cells surrounding a duct collection system. In the mutant mice, fewer collection ducts and dilated renal pelves were observed. Kidneys varied from normal to kidneys in which there was no evident medulla or which contained large cystic-looking regions disrupting the medulla. In general, the degree of disorganization in the medulla was significantly more pronounced than had been observed in the $p57^{Kip2}$ single mutant strain, where slightly fewer collection ducts, less mesenchymal tissue and a reduced medulla as well as more stromal cells were reported (120).

There was a strong correlation between the severity of the placental and kidney phenotypes within individual *p57H19* mutant embryos. It is possible that the disorganized placenta, through ineffective nutrient transfer, results in the defects in kidney development. Alternatively, both tissues might independently be sensitized to excess IGFII in the absence of $p57^{Kip2}$ expression. The variability in the kidney phenotype was observed between animals, not between the two kidneys of a single animal, suggesting that genetic background could play a critical role in its severity.

Renal dysplasia is a common feature of BWS. Specifically, patients with a reduction in the collecting ducts and numerous fluid filled cysts as well as hypertrophy of the mesenchymal tissue have been reported. In humans, renal dysplasia is thought to result from abnormal metanephric differentiation (123). We did not observe hypertrophy in *p57H19* kidneys, which may reflect the fact that most BWS kidneys analyzed are from adults, not fetuses. In all other respects, *p57H19* dysplastic kidneys appear to mimic most of the aspects of the human disease. In contrast to the kidney dysplasias, we saw no defects in adrenal architecture or size in *p57H19* mice. This was surprising in light of the previous finding of adrenomegaly in $p57^{Kip2}$ mice (120).

F. Abdominal Wall Defects

Closure of the ventral abdominal wall in mice occurs around 16.5 d.p.c. and is preceded by the retraction of the midgut into the abdominal cavity. Failure of the intestine to retract results in an umbilical hernia whereas failure of the abdominal wall to close results in an omphalocele, in which the midgut protrudes from the abdomen. Around 60% of BWS patients need surgical correction of omphalacele at birth, andanother 32% display umbilical hernias (Table 3). One or the other of these phenotypes was seen in 55% of *p57H19* embryos, consistent with the previous report that $p57^{Kip2}$ mutants display omphalocele as well as body wall muscle dysplasia. Although this condition is not observed in *H19*Δ13 mutants, it is seen in *Igf2r* and *Igf2r/ H19*Δ13 double mutants, where IGFII levels are elevated >2-fold (115,116,117,114).

G. Cleft Palate and Skeletal Abnormalities

BWS patients occasionally display cleft palate, a condition that results from the failure of the palatal shelves to elevate, rotate or fuse. Previous studies had detected cleft palate in *Igf2r/H19Δ13* as well as in $p57^{Kip2}$ mutant mice but not in either *H19Δ13* or *Igf2r* mutant mice, implying that either significantly elevated IGFII or reduced p57 could lead to the developmental defect. In *p57H19* mutants, the frequency and severity of cleft palate, which was detected in 26% of offspring, was similar to that seen in $p57^{Kip2}$ single mutants (Table 3), suggesting that the primary cause of cleft palate is loss of $p57^{Kip2}$ function. It has been proposed that the cleft palate in $p57^{Kip2}$ mutants results from the failure of $p57^{Kip2}$ mutant cells to exit the cell cycle, and to undergo apoptosis (119).

Polydactyly has been observed in ~5% of BWS patients (Table 3). This phenotype has also been observed in mice displaying elevated IGFII, with the frequency and severity increasing with the dosage of IGFII. $p57^{Kip2}$ single mutants do not display polydactyly whereas *H19Δ13* mice do display postaxial polydactyly 68% of the time on a 129/Sv background (114). As expected for an *Igf2*-dependent phenotype, *p57H19* double mutants also show postaxial polydactyly in two genetic backgrounds examined (Table 4).

H. The Role of Igf2 in the p57H19 phenotype

The dramatic increase in the severity of kidney and placental dysplasia in *p57H19* double mutants occurred in animals over-expressing IGFII. Although the somatic overgrowth observed in *H19Δ13* mutant animals was genetically shown to be the consequence of the over-production of IGFII (113), it is possible that the novel defects observed in *p57H19* mice are due to effects of the *H19* deletion on genes other than *Igf2* or possibly even due to the loss of the *H19* mRNA itself. If the new phenotypes are due to IGFII overproduction, a reduction in IGFII levels should ameliorate them. To test this, *p57H19* females were crossed to $Igf2^{+/-}$ males with a null allele of *Igf2* (*p57H19; Igf2*) (91). Previous studies had determined that while the expression of *Igf2* mRNA from the maternal chromosome in *H19Δ13* mice in mesodermal tissues such as skeletal muscle and heart was essentially equivalent to that on the paternal chromosome, its derepression was less pronounced in endodermal tissues such as liver (113). Furthermore, the circulating levels of IGFII were only modestely elevated (114). Thus overall maternal expression of IGFII in *p57H19; Igf2* mutants is lower than that from a wild-type paternal chromosome.

At 18.5 d.p.c. triple mutant fetuses (*p57H19; Igf2*) were indistinguishable in size from wild-type, or *p57H19* fetuses (Table 2). In contrast, $Igf2^{+/-}$ offspring were 58% the size of wild-type, as had been observed previously (91). Thus in the absence of $p57^{Kip2}$, prenatal growth was relatively insensitive to the ~2-fold differences in the levels of IGFII between *p57H19* and *p57H19;Igf2* mice. The complete absence of IGFII in $p57^{Kip2\ -/+}$; $Igf2^{+/-}$ double mutants, on the other hand, results in embryos that are the same size as their $Igf2^{+/-}$ litter-mates (data not shown).

In contrast to the insensitivity of somatic growth to the reduction in *Igf2*, the placental overgrowth and dysplasia in *p57H19* embryos were almost completely suppressed in triple mutants (Table 2). That is, the mean weight of placentas did not differ from that of wild-type littermate placentas, but did differ substantially from that of the *p57H19* placentas, which were 53% over-sized in this cross. On histological examination, triple mutant placentas were morphologically normal, lacking even the moderate level of disorganization seen in $p57^{Kip2}$ single mutants. Thus the placental dysplasia, which is $p57^{Kip2}$-dependent, can be suppressed by reducing the levels of IGFII.

The same situation appears to hold for the kidney dysplasia. None of the triple mutants had cystic kidneys, and the only discernible differences observed in a few fetuses were a slightly less developed medulla and a slight increase in mesenchymal tissue between the renal tubules. However, inmost animals, the kidneys were less affected than in p57KIP2 animals. Finally, the macroglossia observed in the p57H19 mutant was completely absent in the triple mutant, indicating that overgrowth of the tongue is also Igf2 dependent (Table 2). This is consistent with the finding of macroglossia in Ifg2 overexpressing transgenic mice (118).

The frequency of abdominal wall defects in *p57H19* mice was unaffected by reducing the levels of *Igf2* expression in *p57H19; Igf2* mice (Table 4). This result is somewhat surprising, as omphalocele was induced in the presence of highly elevated levels of IGFII in *Igf2r* and *Igf2r/H19* mutant mice. Thus, in *p57H19* mice, this defect appears to be entirely attributable to *p57*^{Kip2} loss-of-function. Likewise the frequency of cleft palate is not affected by a loss of *Igf2* in the triple mutant, even though cleft palate was observed in *Igf2r/H19* double mutants. Lastly the postaxial polydactyly that is observed in both p*57H19* and *H19* mutants was completely rescued by the reduction in *Igf2* expression.

Aim 4. Analysis of the QT domain.
We screened for proteins that bind to C-terminus of p57 by the two hybrid system. We did not find any specific interactors in this screen and have discontinued them.

Aim 5. Transcriptional control of p57^{KIP2}.
We completed this aim and discussed it in last years report.

Aim 6. Identification of new CKIs and other potential regulators of Cdks from normal breast. This aim proposes to look for additional Cdk binding proteins in the breast using the two hybrid system and breast cDNA libraries. Our screens did not turn up any new proteins that were not already identified in previously published screens usinglibraries from other tissue sources.

Conclusion:

The last year was a very productive one for our lab and the cell cycle field in general. Our work funded under this grant allowed us to establish the role of p57^{KIP2} in mouse development and the human cancer and overgrowth syndrome BWS. p57^{KIP2} clearly acts as a regulator of cell proliferation in the adrenal gland, the lens epithelia, and certain chondrocytes. The partial dependency on p57^{KIP2} for reducing cell proliferation reveals the redundant mechanisms used to limit tissue growth. A similar situation is observed in cell culture where agents that induce cell cycle arrest immediately increase levels of certain CKIs and subsequently reduce the levels of the cyclins and Cdks. While undergoing the process of reducing Cdk activity during differentiation, the absence of CKIs may allow additional cell cycles to occur before Cdk activity is sufficiently reduced to block cell cycle entry. In addition, other CKIs may provide Cdk inhibitory functions in the absence of p57 ^{KIP2}, as we have shown here in the lens development of p27/p57 double mutant mice.

CKIs are the ultimate effectors of signal transduction pathway intended to bring about cell cycle arrest and the patterns of expression during embryonic development suggest that particular *CKIs* play important roles in terminal differentiation in a tissue specific manner. However, the fact that mice lacking single *CKIs* display surprisingly few developmental phenotypes has brought into question the essential nature of *CKIs* for cell cycle arrest and differentiation. Our studies **funded by this grant demonstrate** that two *CKIs*, *p57* and *p21* cooperate to control proliferation and differentiation in multiple tissues and reiterates the critical importance of *CKIs* to cell cycle control during development. The use of multiple *CKIs*, each controlled through distinct signaling pathways, provides a flexible mechanism to control proliferation in a cell type specific manner. It is likely that the combinatorial use of *CKIs* will emerge as one of the principal means through which cell cycle arrest and differentiation are integrated during development.

The more recent studies on the roles of IGF2 and p57 was prompted by the clinical findings in BWS that mutations in *p57*^{KIP2} and over-expression of *IGF2* have each been proposed as causes of the disease. Yet the phenotypes of the mouse models for loss of function of p57KIP2 and loss of

imprinting of Igf2 are readily distinguished (113, 119, 120, 124) McLaughlin et al (124) have shown that mice containing a UPD of a large portion of the distal end of mouse chromosome 7 die at 9.5 d.p.c., presumably as a consequence of the reduced expression of *Mash2,* a gene that is required for placental development and is maternally expressed (125, 126). By creating a mouse model in which only the two candidate genes are affected, we hoped to gain more precise insight into the ways in which mis-regulation of these two apparently unrelated genes could lead to the same disease.

The *p57H19* mice exhibited a dramatic increase in the severity of several BWS phenotypes such as placental overgrowth and dysplasia and kidney defects. Furthermore, macroglossia, one of the hallmarks of BWS, was seen in these mice. A direct interaction between the $p57^{Kip2}$ and *Igf2* pathways is implied by the ability of the *Igf2* mutation to compensate for the placental and kidney dysplasias that arise from mutations in $p57^{Kip2}$. Thus in the absence of $p57^{Kip2}$, the disorganized development of those tissues is enhanced in an *Igf2*-dependent manner. When signaling through *Igf2* is reduced, the loss of $p57^{Kip2}$ is no longer detrimental. Thus our analysis of *p57H19* mice suggests a resolution to the dilemma of how both loss-of-function mutations in $p57^{KIP2}$ and gain-of function mutations in *IGF2* can lead to BWS. That is, in the mouse the two genes act in an antagonistic manner in a subset of the tissues in which they are co-expressed.

A pathway that is affected by both genes is the one that regulates G1 cell cycle progression. Both IGFII and p57 are involved in regulating the progression of cells through the G1/S phase of the cell cycle, with IGFII promoting the G1/S transition (127) and p57 inhibiting the G1 cyclin-dependent kinases (CDKs) (89, 90). p57 has not been implicated in regulating embryo size, but is involved in the cell cycle arrest that precedes terminal differentiation of tissues such as skeletal muscle, lens and placenta (120, 121, 128). IGFII, on the other hand, is a direct regulator of fetal growth, and has been shown to promote progression through the G1 phase of the cell cycle, possibly through its ability to increase the level of the G1 cyclin D1 (127). The decision to proceed through the G1 to S phase transition checkpoint is controlled by the ratio of cyclin/Cdk complexes to CKIs, which determines the overall activity of G1-cyclin/Cdk complexes. It is possible that the exacerbation of the $p57^{Kip2}$ phenotype in the presence of excess IGFII and its alleviation when the concentration of the growth factor is reduced reflects cell-type specific sensitivity to Cdk activity in placenta and kidney. Excess IGFII in the absence of $p57^{Kip2}$ could lead to hyperproliferation, as seen in the placenta, to increased apoptosis, which is observed in both $p57^{Kip2}$ and *p57H19* mutant placentas and kidneys, or to a failure to differentiate, as is suggested by the reduction of medullary cells in the kidney. Increased apoptosis has also been reported in the palatal shelves of $p57^{Kip2}$ single mutants, and presumably results from alterations in the orderly progression through cell cycle checkpoints (119).

Several $p57^{Kip2}$-dependent phenotypes such as cleft palate and omphalocele are neither enhanced by over-expression of *Igf2*, nor rescued by its absence. It may be that the insensitivity of cleft palate and omphalocele phenotypes to changes in IGFII levels reflects the fact that these tissues regulate the CDK to CKI levels using growth factors other than IGFII. On the other hand both cleft palate and omphalocele are observed in the most severe IGFII gain-of-function mouse model, the *Igf2r/*H19 double mutant, where IGFII levels were 7-11 fold higher than normal (114). These animals also displayed the lens abnormalities and skeletal defects that were seen in the $p57^{Kip2}$ mutants but are not associated with BWS. Thus it may be that in the presence of $p57^{Kip2}$, very high levels of IGFII are required to alter the CDK to CKI balance.

One of the surprising findings in this study was that the somatic overgrowth that has been shown to be *Igf2*-dependent in *H19Δ13* mice was less pronounced in *p57H19* mice. We considered the possibility that the lack of somatic overgrowth was due to the failure of the dysplastic placenta to provide nutrients to the embryo in the later stages of gestation. However the subset of *p57H19* mice in which the placenta was relatively normal were not oversized at birth (data not shown). Furthermore there was a modest positive correlation between the degree of placental hyperplasia and the size of the embryos in general (r=0.37). The fact that $p57^{Kip2}$ mutants are reduced in size in the presence of a null mutation in *Igf2* argues that the $p57^{Kip2}$ mutation does not completely desensitize the embryo to

changes in IGFII concentration. Rather it may be that the~2-fold changes in IGFII expression between *p57*^{Kip2}, *p57H19* and *p57H19; Igf2* mice do not shift the ratios of CDKs to CKIs sufficiently to effect a change in overall growth rate. Of the Cip/Kip family of CKIs, only *p27*^{Kip1} has been directly implicated in overall fetal growth (129, 130, 131, 132).

Our results suggest a model in which p57 and IGFII act antagonistically in the control of cell proliferation and development in several tissues affected in BWS patients including the tongue, kidney, and placenta. The affected tissues in the mouse are those in which some rate-limiting step is controlled by the two growth regulators, while other tissues, like the liver, presumably utilize other positive or negative growth signals. Although the nature of these other pathways are unknown, IGFI has been shown to function redundantly with IGFII to promote cell growth (133). Likewise several instances of redundancy between Cdk inhibitors have been observed. For example, p21 and p57 are redundant for control of muscle and lung development (128); p27 and p57 are redundant for control of lens development (121); and p18 and p27 work together to control pituitary gland, spleen, and thymus embryonic growth (134). We propose that BWS phenotypes are observed when the overall balance of regulators are shifted in favor of proliferation by either an increase in IGFII, or a decrease in p57. While a shift in the balance of other regulators could potentially cause a BWS-like phenotype, it is likely that the differing tissue specificity of the other regulators would prevent them from producing a phenotype recognized as BWS.

The fact that the *p57H9* double mutant does not completely recapitulate the BWS phenotype probably reflects species-specific differences in the tissues in which the two genes act directly in opposition to one another. Furthermore because the changes in the cell number in a given organ due to a change in the rate of proliferation is an exponential function, the more rounds of cell division a tissue undergoes, the greater will be the effect of a small increase in proliferation rate. Since human organs such as the tongue, kidney and placenta undergo significantly more cell division, a relatively small change in proliferation rates afforded by p57 loss or increased IGFII may have a more pronounced effect in humans relative to the mouse.

The variability in the phenotypes in the *p57H19* mice is reminiscent of the highly variable phenotypes of BWS patients (Table 3). The mice in this study were not on completely inbred backgrounds, and at least some of the variability we observed could be attributed to genetic modifiers. Another explanation for the variability in the human syndrome is suggested by the curious clinical finding that 10 sets of female identical twins have been reported who are discordant for BWS (135). Although the high level of discordancy is not understood, it has been suggested that disruptions in epigenetic mechanisms such as X-inactivation and imprinting might explain the occurrence, an explanation that could extend to sporadic cases as well.

In conclusion the analysis of the defects in *p57H19* mice demonstrates that some but not all tissues are highly sensitive to the ratio of p57 and IGFII. Perturbations in the levels of either protein may be sufficient to generate the variable range of phenotypes in BWS.

References

1. Ohtsubo, M., and Roberts, J.M. (1993). Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science *259*, 1908-1912.
2. Dou, Q.-P., Levin, A.H., Zhao, S., and Pardee, A.B. (1993). Cyclin E and Cyclin A as candidates for the restriction point protein. Cancer Res. *53*, 1493-1487.
3. Quelle, D.E., Ashmun, R.A., Shurtleff, S.A., Kato, J., Bar-Sagl, D., Roussel, M.F., and Sherr, C.J. (1993) Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev. 7, 1559-1571.
4. Pardee, A.B. (1989). G1 events and regulation of cell proliferation. Science 246, 603-608.
5. C. Koch, C. and Nasmyth, K. (1994) Cell cycle regulated transcription in yeast. Curr. Opin. Cell Biol. 6: 451-459
6. Sherr, C.J. (1994) G1 Phase progression: Cycling on cue. Cell 79, 551-555.
7. Hunter, T., and Pines, J. (1994) Cyclins and Cance II: Cyclin D and CDK inhibitors come of age. Cell 79, 573-582.
8. Meyerson, M., Enders, C.-L., Wu, L.-K., Su, C., Gorka, C., Neilson, E., Harlow, E., and Tsai, L.-H. (1992) A Family of human cdc2-related protein kinases. EMBO J. 11, 2909-2917.
9. Nurse, P. (1994) Ordering S phase and M phase in the cell cycle. Cell 79, 547-550.
10. Draetta, G.F. (1994) Mammalian G1 cyclins. Curr. Opin. Cell. Biol. 6, 842-846.
11. King, R.W., Jackson, P.K., and Kirschner, M.W. (1994) Mitosis in transition. Cell 79, 563-571.
12. Coleman, T.R., an Dunphy, W.G. (1994) Cdc2 regulatory factors. Cur. Opin. Cell Bio. 6, 877-882.
13. Matsushime, H., Roussel, M.F., Ashmun, R.A., and Sherr, C.J. (1991). Colony stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell *65*, 701-713.
14. Koff, A., Giordano, A., Desia, D., Yamashita, K., Harper, J.W., Elledge, S.J., Nishimoto, T., Morgan, D.O., Franza, R., and Roberts, J.M. (1992). Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science *257*, 1689-1694.
15. Dulic, V., Lees, E., and Reed, S.I. (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. Science *257*, 1958-1961.
16. Baldin, V., Lukas, J., Marcote, M.J., Pagano, M., and Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. *7*, 812-21.
17. Resnitzky, D., and Reed, S.I. (1995) Different roles for cyclins D1 and E in regulation of theG1 to S transition. Mol. Cell. Biol. 15, 3463-3469.
18. Resnitzky, D., Hengst, L., and Reed, S.I. (1995) Cyclin A-Associated kinase activity is rate limiting for entrance is not S-phase and is negatively regulated in G1 by p27KIP1. Mol. Cell. Biol. 15, 4347-4352.
19. Keyomarsi, K., and Pardee, A.B. (1993). Redundant cyclin overexpression and gene amplification in breast cancer cells. Proc. Natl. Acad. Sci. USA *90*, 1112-1116.
20. Leach, F.S., Elledge, S.J., Sherr, C.J., Willson, J.K.V., Markowitz, S., Kinzler, K.W., and Vogelstein, B. (1993). Amplification of cyclin genes in colorectal carcinomas. Cancer Research *53*, 1986-1989.
21. Lammie, G.A., Fantl, V., Smith, R., Schuuring, E., Brookes, S., Michalides, R., Dickson, C., Arnold, A., and Peters, G. (1991). D11S287, a putative oncogene on chromosome 11g13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. Oncogene *6*, 439-444.
22. Seto, M., Yamamoto, K., Lida, S., Akoa, Y., Utsumi, K., Kubonishi, I., Miyoshi, I., Ohtsuki, T., Yawata, Y. Namba, M., Motokura, T., Arnold, A., Takahashi, T., and Ueda, R. (1992) Gene rearrangement and overexpression of PRAD1 in lymphoid malignancy with t(11;14)(q13;q32) translocation. Oncogene *7*, 1401-1406.

23. Xiong, Y., Mennenger, J., Beach, D., and Ward, D.C. (1992). Molecular cloning and chromosomal mapping of CCND genes encoding human D-type cyclins. Genomics *13*, 575-584.
24. Motokura, T., Bloom, T., Kim, H.G., Juppner, H., Ruderman, J.V., Kronenberg, H.M., and Arnold, A. (1991). A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature *350*, 512-515.
25. Wang, J., Chenivesse, X., Henglein, B., and Brechot, C. (1990). Hepatitis B virus integration in a cyclin A gene in a hepatocarcinoma. Nature *343*, 555-557.
26. Ewen, M.E., Sluss,, H.K., Sherr, C.J., Matsushime, H., Kato, J.Y., and Livingston, D.M (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell 73, 487-497.
27. Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., and Sherr, C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (Rb) and pRb phosphorylation by the cyclin D-dependent protein kinase Cdk4. Genes Dev. 7, 331-342.39.
28. Hinds, P.W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S.I., and Weinberg, R.A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell *70*, 993-1006.
29. Weinberg, R.A. (1995) The retinoblastoma protein and cell cycle control. Cell 81, 323-330.
30. Pagano, M., Pepperkok, J., Lukas, V., Baldin, W., Ansorge, J., Bartek, ., and Draetta, G. (1993). Regulation of the human cell cycle by the cdk2 protein kinase. J. Cell. Biol. *121*, 101-111.
31. van der Heuvel, S., and E. Harlow. (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* 26: 2050-2054.
32. Fang, F., and Newport, J.W. (1991). Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in eukaryotes. Cell *66*, 731-742.
33. Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J. and Pagano, M. (1995) Human cyclin E, a nuclear protein essential for the G1/S phase transition. Mol. Cell. Biol. 15, 2612-2624.
34. Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draeta, G. (1992) Cyclin A is Required at Two Points in the Human Cell Cycle. EMBO J. 11, 961-971.
35. Zindy, F., Lamas, E., Chenivesse, X., Sobczak, J., Wang, J., Fesquet, D., Henglein,B., and Brechot, C. (1992) Cyclin A is Required in S Phase in Normal Epithelial Cells. Biochem. Biophys. Res. Commun. 182, 1144-1154.
36. Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N.J.C. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell *67*, 1169-1179.
37. Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.S., Elledge S.J., and R.A. Weinberg. (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and the breast. Cell 82, 621-630.
38. Cox, L.S., and Lane, D.P. (1995). Tumor suppressors, kinases and clamps: how *p53* regulates the cell cycle in response to DNA damage. BioEssays *17*, 501-508.
39. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991) p53 Mutations in Human Cancers. Science 253, 49-53.
40. T'ang, A., Varley, J.M., Chakraborty, S., Murphee, A.L., and Fung, Y.-K.T. (1988) Structural rearrangement of the retinoblastoma gene in human breast cancer. Science 242, 263-266.
41. Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimchi, O., and Oren, M. (1989) Wild-Type p53 Can Inhibit Oncogene-Mediated Focu Formation. Proc. Natl. Acad. Sci. USA 86, 8763-8767.
42. Finlay, C.A., Hinds, P.W., and Levin, A.J. (1989) The p53 Proto-oncogene Can Act as a Suppressor of Transformation. Cell 57, 1083-1093.
43. Rotter, V., Foord, O., and Narot, N. (1993) In search of the function of normal p53 protein. Trends Cell Biol. 3, 46-49.

44. Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J.S., and Bradley, A. (1992) Mice Deficient in p53 are Developmentally normal but Susceptible to Spontaneous Tumors. Nature 356, 215-221.
45. Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V., and Kastan, M.B. (1992) Wild-type p57 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA 89, 7491-7495.
46. Weinert, T., and Lydall, D. (1993) Cell cycle checkpoints, genetic instability and cancer. Semin. Cancer Biol. 4, 129-140.
47. Hartwell, L. (1992) Defects in a cell cycle checkpoint may be responsible for the genetic instability of cancer cells. Cell 71, 543-546.
48. Zhan, Q., Carrier, F., and Fornace, A.J. (1993) Induction of cellular p53 activity by DNA damaging agents and growth arrest. Mol. Cell. Biol. 13, 4242-4250.
49. Kastan, M.B., Zhan, Q., El-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett checkpoint pathway utilizing p53 and GADD45 is defective in ataxia telangiectasia. Cell 71, 587-597.
50. Oren, M. (1992) The involvement of oncogenes and tumor suppressor genes in the control of apoptosis. Cancer Metastasis Rev. 11, 141-148.
51. Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. (1992) Induction apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA 89, 4495-4499.
52. Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L.,and Wyllie, A.H. (1993) Thymocyte apoptosis induced by p53 dependent and independent pathways. Nature 362, 849-852.
53. Hartwell, L. H. and Kastan, M. B. (1994). Cell cycle control and cancer. Science *266*, 1821-1828.
54. Cross, S. M. , Sanchez, C. A., Morgan, C. A. , Schimke, M. K., Ramel, Idzerda, R. L., Raskind, W. H. & B.J. Reid. (1995). A *p53*-dependent mouse spindle checkpoint. Science *267*, 1353-1356.
55. Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tisty, T.D. (1992) Altered Cell Cycle Arrest and Gene Amplification Potential Accompany Loss of Wild-Type p53. Cell 70, 923-935.
56. Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P.J. (1995) Mice lacking *p21CIP1/WAF1* undergo normal development, but are defective in G1 checkpoint control. Cell 82:675-684.
57. Harper, J.W., Adami, G., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993) The 21 kd Cdk interacting protein is a potent inhibitor of G1 cyclin-dependent kinases. Cell,75:805-816.
58. Xiong, Y., G. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. Nature 366, 701-704.
59. El-Deiry, W.S., Tokino, T., Velculesco, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75, 817-825.
60. Noda, A., Y. Ning, S.F. Venable, O.M. Pereira-Smith, and J.R. Smith. (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression cloning screen. *Exp. Cell Res.* 211: 90-98.
61. Serrano, M., G.J. Hannon, and D. Beach. (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366: 704-707.
62. Hannon, G.J., and Beach, D. (1994) $p15^{INK4B}$ is a potential effector of TGF-b induced cell cycle arrest. Nature 371, 257-261.
63. Jen, J., J.. Harper, S.H. Bigner, D.D. Bigner, N. Papadopoulos, S. Markowitz, J.K.V. Willson, K.W. Kinzler, and B. Vogelstein. (1994) Deletion of p16 and p15 genes in brain tumors. Cancer Res. 54, 6353-6358.

64. Guan, K., C.W. Jenkins, Y. Li, M.A. Nichols, X. Wu, C.L. O'keefe, A.G. Matera, and Y. Xiong. (1994) Growth suppression by p18, a $p16^{INK4/MTS1}$ and $p14^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. Genes and Dev. 8, 2939-2952.
65. Hirai, H., Roussel, M.F., Kato, J.Y., Ashmun, R.A., and Sherr, C. (1995) Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin dependent kinases Cdk4 and Cdk6. Mol. Cell. Biol. 15, 2672-2681.
66. Polyak, K., M.H. Lee, H. Erdjument-Bromage, P. Tempst, and J. Massagu. (1994) Cloning of $p27^{KIP1}$, a cyclin-dependent kinase inhibitor and potential mediator of extracellular antimitogenic signals. Cell 78, 59-66.
67. Toyoshima, H. and T. Hunter. (1994) p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 78, 67-74.
68. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995) Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGFβ. Genes Dev. 9, 1831-1845.
69. Matsuoka, S., Edwards, M., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J.W., and S.J. Elledge. (1995) $p57^{KIP2}$, a structurally distinct member of the $p21^{CIP1}$ Cdk-inhibitor family, is a candidate tumor suppressor gene. Genes and Dev. 9, 650-662.
70. Seizinger, B., H. P.Klinger, C. Junien, Y. Nakamura, M. Le Beau, W. Cavenee, B. Amanuel, B. Ponder, S. Naylor, F. Mittelman, D. Louis, A. Menon, I. Newsham, J. Decker, M. Kaelbling, I. Henry, and A. V. Deimling. (1991) Report of the committee on chromosome and gene loss in human neoplasia. *Cytogenet. Cell. Genet.* 58:1080-1096.
71. Hastie, N.D. (1994) The Genetics of Wilm's tumor. A case of disrupted development. Ann. Rev.*Genetics* 28: 523-558.
72. Wiedemann, H. R. (1983) Tumours and hemihypertrophy associated with Wiedemann-Beckwith syndrome. *Eur. J. Pediatr.* 141:129.
73. Pettenati, M. J., J. L. Haines, R. R. HIggins, R. S. Wappner, C. G. Palmer, and D. Weaver. (1986) Beckwith-Wiedemann syndrome: Presentation of Clinical and cytogenetic date on 22 new cases and review of the literature. *Hum. Genet.* 74: 143-154.
74. Junien, C. (1992) Beckwith-Wiedemann Syndrome, tumourigenesis and imprinting. *Current Opinion in Genetics and Development*. 2: 431-438.
75. Negrini, M., Rasio, D., Hampton, G.M., Sabbioni, S., Rattan, S., Carter, S.L., Rosenberg, A.L., Schwartz, G. F., Shiloh, Y., Cavenee, W.K. (1995) Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3. Cancer Res 55: 3003-3007.
76. Zhang, P., Liegeois, N.J., Wong, C., Hou, H., Finegold, M., DeMayo, F., Silverman, A., Harper, J.W., DePinho, R.A., and Elledge, S. J.(1997). Altered cell differentiation and proliferation in mice lacking $p57^{KIP2}$ indicates a role in Beckwith-Wiedeman Syndrome. *Nature* 387:151-158.
77. Yan, Y., J. Frisen, M.H. Lee, J. Massague and M. Barbacid. 1997. Ablation of the CDK inhibitor *p57KIP2* results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* **11:** 973-983.
78. Hasty, P., A. Bradley, J.H. Morris, D.G. Edmondson, J.M. Venuti, E.N. Olson, and W.H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene [see comments]. *Nature* **364**: 501-506.
79. Nabeshima, Y., K. Hanaoka, M. Hayasaka, E. Esumi, S. Li, and I. Nonaka. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect [see comments]. *Nature* **364**: 532-535.
80. Kelly, A.M. 1983. Emergence of specialization of skeletal muscle. In *Handbook of Physiology* (ed. L.D. Peachey), pp. 507-537. Williams & Williams Co., Baltimore, MD.

81. Venuti, J.M., J.H. Morris, J.L. Vivian, E.N. Olson, and W.H. Klein. 1995. Myogenin is required for late but not early aspects of myogenesis during mouse development. *J Cell Biol* **128**: 563-576.
82. Parker, S.B., G. Eichele, P. Zhang, A. Rawls, A.T. Sands, A. Bradley, E.N. Olson, J.W. Harper, and S.J. Elledge. 1995. p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells [see comments]. *Science* **267**: 1024-1027.
83. Elias, E. R., DeBaun, M. R. and Feinberg, A. P. 1998. Beckwith-Wiedemann Syndrome. Principles of Molecular Medicine. Humana Press Inc. 1047-1052.
84. Koufos, A., Grundy, P., Morgan, K., Aleck, K. A., Hadro, T., Lampkin, B. C., Kalbakji, A. and Cavenee, W. 1989. Familial Wiedemann-Beckwith syndrome and a second Wilms tumor locus both map to 11p15.5. *Am. J. Hum. Gen.* **44**: 711-719.
85. Ping, A. J., Reeve, A. E., Law, D. J., Young, M. R., Boehnke, M. and Feinberg, A. P. 1989. Genetic Linakge of Beckwith-Wiedemann syndrome to 11p15. *Am. J. Hum. Genet.* **44**: 720-723.
86. Turleau, C., deGrouchy, J., Chjavin-Colin, F., Martelli, H., Voyer, M. and Charlas, R. 1984. Trisomy 11p15 and Beckwith-Wiedemann syndrome: a report of two cases. *Hum. Genet,* **67**: 219-221.
87. Henry, I., Bonalti-Pellie, C., Chehensse, V., Beldjord, C., Schwartz, C., Utermann, G. and Junien, C. 1991. Uniparental paternal disomy in a genetic cancer-predisposing syndrome. *Nature* **351**: 665-667.
88. Lee, J. E., Pintar, J. and Efstratiadis, A. 1990. Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. *Development* **110**: 151-159.
89. Lee, M. H., Reynisdottir, I. and Massague, J. 1995. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* **9**(6): 639-49.
90. Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W. and Elledge, S. J. 1995. p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev* **9**(6): 650-62.
91. DeChiara, T. M., Efstratiadis, A. and Robertson, E. J. 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345**: 78-80.
92. Giannoukakis, N., Deal, C., Paquette, J., Goodyer, C. G. and Polychronakos, C. 1993. Parental genomic imprinting of the human *IGF2* gene. *Nat. Genet.* **4**(1): 98-101.
93. Ohlsson, R., Nystrom, A., Pfeifer-Ohlsson, S., Tohonen, V., Hedborg, F., Schofield, P., Flam, F. and Ekstrom, T. J. 1993. IGF2 is parentally imprinted during human embryogenesis and in the Beckwith-Wiedemann syndrome. *Nat. Genet.* **4**(1): 94-7.
94. Weksberg, R., Shen, D. R., Fei, Y. L., Song, Q. L. and Squire, J. 1993. Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nat. Genet.* **5**: 143-150.
95. Reik, W., Brown, K. W., Schneid, H., LeBouc, Y., Bickmore, W. and Maher, E. R. 1995. Imprinting mutations in the Beckwith-Wiedemann syndrome suggested by an altered imprinting pattern in the *IGF2-H19* domain. *Hum. Mol. Genet.* **4**: 2379-2385.
96. Hatada, I., Inazawa, J., Abe, T., Nakyama, M., Kaneko, Y., Jinno, Y., Niikawa, N., Ohashi, H., Fukushima, Y., Iida, K., Yutani, C., Takahashi, S., Chiba, Y., Ohishi, S. and Mukai, T. 1996. Genomic imprinting of human *p57^KIP2^* and its reduced expression in Wilms' tumors. *Hum. Mol. Genet.* **6**: 783-788.
97. O'Keefe, D., Dao, D., Zhao, L., Sanderson, R., Warburton, D., Weiss, L., Anyane-Yeboa, K. and Tycko, B. 1997. Coding mutations in *p57KIP2* are present in some cases of Beckwith-Wiedemann syndrome but are rare or absent in Wilms tumors. *Am. J. Hum. Genet.* **61**: 295-303.
98. Lam, W.W., Hatada, S., Ohishi, T., Mukai, J.A., Joyce, T.R., Cole, D. Donnai, W. Reik, P.N. Schofield, and E.R. Maher. 1999. Analysis of germling CDKN1C 9p57KIP2)

mutations in familial and sporadic Beckwith-Wiedemann syndrome (BWS) provides a novel genotype/phenotype correlation. J. Med. Genet. 7:518-523.
99. Hatada, I. and Mukai, T. 1995. Genomic imprinting of *p57/KIP2*, a cyclin-dependent kinase inhibitor, in mouse. *Nat. Genet.* **11**: 204-206.
100. Matsuoka, S., Thompson, J. S., Edwards, M. C., Barletta, J. M., Grundy, P., Kalikin, L. M., Harper, J. W., Elledge, S. J. and Feinberg, A. P. 1996. Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, p57KIP2, on chromosome 11p15. *Proc. Nat. Acad. Sci. USA* **93**: 3026-3030.
101. Hatada, I., Ohashi, H., Fukushima, Y., Kanetko, Y., Inoue, M., Komoto, Y., Okada, A., Ohishi, S., Nabetani, A., Morisaki, H., Nakayama, M., Niikawa, N. and Mukai, T. 1996. An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. *Nat. Genet.* **14**: 171-173.
102. Lee, M. P., Hu, R., Johnson, L. A. and Feinberg, A. P. 1997. Human *KVLQT1* gene shows tissue-specific imprinting and encompasses Beckwith-Wiedemann syndrome chromosomal rearrangements. *Nat. Genet.* **15**: 181-185.
103. Caspary, T., Cleary, M. A., Baker, C. C., Guan, X.-J. and Tilghman, S. M. 1998. Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. *Mol. Cell. Biol.* **18**(6): 3466-3474.
104. Paulsen, M., K.R. Davies, L.M. Bowden, A.J. Villar, O. Fracnk, M. Fuermann, W.L. Dean, Moore, T. F., Rodrigues, N., Davies, K. E., Hu, R. J., Feinberg, A. P., Maher, E. R., Reik, W. and Walter, J. 1998. Syntenic organization of the mouse distal chromosome 7 imprinting cluster and the Beckwith-Wiedemann syndrome region in chromosome 11p15.5. *Hum Mol Genet* **7**(7): 1149-59.
105. Weksberg, R., Teshima, I., Williams, B. R., Greenberg, C. R., Pueschel, S. M., Chernos, J. E., Fowlow, S. B., Hoyme, E., Anderson, I. J., Whiteman, D. A. and et, a. 1993. Molecular characterization of cytogenetic alterations associated with the Beckwith-Wiedemann syndrome (BWS) phenotype refines the localization and suggests the gene for BWS is imprinted. *Hum. Mol. Genet.* **2**(5): 549-56.
106. Mannens, M., Hoovers, J. M., Redeker, E., Verjaal, M., Feinberg, A. P., Little, P., Boavida, M., Coad, N., Steenman, M., Bliek, J. and et al. 1994. Parental imprinting of human chromosome region 11p15.3-pter involved in the Beckwith-Wiedemann syndrome and various human neoplasia. *Eur J Hum Genet* **2**(1): 3-23.
107. Hoovers, J. M., Kalikin, L. M., Johnson, L. A., Alders, M., Redeker, B., Law, D. J., Bliek, J., Steenman, M., Benedict, M. and Wiegant, J. 1995. Multiple genetic loci within 11p15 defined by Beckwith-Wiedemann syndrome rearrangement breakpoints and subchromosomal transferable fragments. *Proc. Nat. Acad. Sci. USA* **92**: 12456-12460.
108. Wang, Q., Curran, M. E., Splawski, I., Burn, T. C., Millholland, J. M., VanRaay, T. J., Shen, J., Timothy, K. W., Vincent, G. M., de Jager, T., Schwatz, P. J., Towbin, J. A., Moss, A. J., Atkinson, D. L., Landes, G. M., Connors, T. D. and Keating, M. T. 1996. Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias. *Nature Genetics* **12**: 17-23.
109. Brown, K. W., Villar, A. J., Bickmore, W., Clayton-Smith, J., Catchpole, D., Maher, E. R. and Reik, W. 1996. Imprinting mutation in the Beckwith-Wiedemann syndrome leads to biallelic *IGF2* expression through an *H19*-independent pathway. *Hum. Mol. Genet.* **5**: 2027-2032.
110. Lee, M. P., DeBaun, M. R., Mitsuya, K., Galonek, H. L., Brandenburg, S., Oshimura, M. and Feinberg, A. P. 1999. Loss of imprinting of a paternally expressed transcript, with antisense orientation to KVLQT1, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. *Proc Natl Acad Sci U S A* **96**(9): 5203-8.
111. Mitsuya, K., Meguro, M., Lee, M. P., Katoh, M., Schulz, T. C., Kugoh, H., Yoshida, M. A., Niikawa, N., Feinberg, A. P. and Oshimura, M. 1999. LIT1, an imprinted

antisense RNA in the human KvLQT1 locus identified by screening for differentially expressed transcripts using monochromosomal hybrids. *Hum Mol Genet* **8**(7): 1209-1217.

112. Smilinich, N. J., Day, C. D., Fitzpatrick, G. V., Caldwell, G. M., Lossie, A. C., Cooper, P. R., Smallwood, A. C., Joyce, J. A., Schofield, P. N., Reik, W., Nicholls, R. D., Weksberg, R., Driscoll, D. J., Maher, E. R., Shows, T. B. and Higgins, M. J. 1999. A maternally methylated CpG island in KvLQT1 is associated with an antisense paternal transcript and loss of imprinting in beckwith- wiedemann syndrome [In Process Citation]. *Proc Natl Acad Sci U S A* **96**(14): 8064-9.
113. Leighton, P. A., Ingram, R. S., Eggenschwiler, J., Efstratiadis, A. and Tilghman, S. M. 1995. Disruption of imprinting caused by deletion of the *H19* gene region in mice. *Nature* **375**: 34-39.
114. Eggenschwiler, J., Ludwig, T., Fisher, P., Leighton, P. A., Tilghman, S. M. and Efstratiadis, A. 1997. Mouse mutant embryos overexpressing IGF-II exhibit phonotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev.* **11**: 3128-3142.
115. Filson, A., Louvi, A., Efstradiatis, A. and Roberston, E. J. 1993. Rescue of the *T-associated maternal effect* in mice carrying null mutations in *Igf-2* and *Igf2r*, two reciprocally imprinted genes. *Develop.* **118**: 731-736.
116. Lau, M. M. H., Stewart, C. E. H., Liu, Z., Bhatt, H., Rotwein, P. and Stewart, C. L. 1994. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev.* **8**: 2953-2963.
117. Wang, Z.-Q., Fung, M. R., Barlow, D. P. and Wagner, E. F. 1994. Regulation of embryonic growth and lysosomal targeting by the imprinted *Igf2/Mpr* gene. *Nature* **372**: 464-467.
118. Sun, F.-L., Dean, W., Kelsey, G., Allen, N. D. and Reik, W. 1997. Transactivation of Igf2 in a mouse model of Beckwith-Wiedemann syndrome. *Nature* **389**: 809-815.
119. Yan, Y., Frisén, J., Lee, M.-H., Massagué, J. and Barbacid, M. 1997. Ablation of the CDK inhibitor p57KIP2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* **11**: 973-983.
120. Zhang, P., Liégeois, N. J., Wong, C., Finegold, M., Hou, H., Thompson, J. C., Silverman, A., Harper, J. W., DePinho, R. A. and Elledge, S. J. 1997. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann sydrome. *Nature* **387**: 151-158.
121. Zhang, P., Wong, C., DePinho, R. A., Harper, J. W. and Elledge, S. J. 1998. Cooperation between the Cdk inhibitors p27KIP1 and p57KIP2 in the control of tissue growth and development. *Genes and Development* **12**: 3162-3167.
122. McCowan, L. M. E. and Becroft, D. M. O. 1994. Beckwith-Wiedemann Syndrome, Placental abnormalities, and gestational proteinuric hypertension. *Obstetrics & Gynecology* **83**(5): 813-817.
123. Oski, F. A., DeAngelis, C. A., Feigin, R. D., McMillan, J. A. and Warshaw, J. B., Ed. 1994. Principles and Practice of Pediatrics. Philadelphia, J.B. Lippincott Company.
124. MacLaughlin, K. J., Szabo, P., Haegel, H. and Mann, J. R. 1996. Mouse embryos with paternal duplication of an imprinted chromosome 7 region die at midgestation and lack placental spongiotrophoblast. *Development* **122**(1): 265-70.
125. Guillemot, F., Nagy, A., Auerbach, A., Rossant, J. and Joyner, A. L. 1994. Essential role of *Mash-2* in extraembryonic development. *Nature* **371**: 333-336.
126. Guillemot, F., Caspary, T., Tilghman, S. M., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Anderson, D. J., Joyner, A. L., Rossant, J. and Nagy, A. 1995. Genomic imprinting of *Mash-2,* a mouse gene required for trophoblast development. *Nature Genet.* **9**: 235-241.
127. Zhang, L., Kim, M., Choi, Y. H., Goemans, B., Yeung, C., Hu, Z., Zhan, S., Seth, P. and Helman, L. J. 1999. Diminished G1 checkpoint after gamma-irradiation and altered

cell cycle regulation by insulin-like growth factor II overexpression. *J Biol Chem* **274**(19): 13118-26.
128. Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J. W. and Elledge, S. J. 1999. p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev* **13**(2): 213-24.
129. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. and Leder, P. 1995. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**(4): 675-84
130. Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Firpo, E., Polyak, K., Tsai, L. H., Broudy, V., Perlmutter, R. M., Kaushansky, K. and Roberts, J. M. 1996. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* **85**(5): 733-44.
131. Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohman, L. A. and Koff, A. 1996. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* **85**(5): 721-32.
132. Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I. and Loh, D. Y. 1996. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**(5): 707-20.
133. Efstratiadis, A. 1998. Genetics of mouse growth. *Int J Dev Biol* **42**(7 Spec No): 955-76.
134. Franklin, D. S., Godfrey, V. L., Lee, H., Kovalev, G. I., Schoonhoven, R., Chen-Kiang, S., Su, L. and Xiong, Y. 1998. CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. *Genes Dev* **12**(18): 2899-911.
135. Clayton-Smith, J., Read, A. P. and Donnai, D. 1992. Monozygotic Twinning and Wiedemann-Beckwith Syndrome. *American Journal of Medical Genetics* **42**: 633-637.
136. DeChiara, T. M., Robertson, E. J. and Efstratiadis, A. 1991. Parental imprinting of the mouse *insulin-like growth factor II* gene. *Cell* **64**: 849-859.
137. Louvi, A., Accili, D. and Efstratiadis, A. 1997. Growth promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev. Biol.* **189**: 33-48.

Appendix

Tables 1-4

Table 1. Genetic crosses

Cross	# litters	Genotype	#embryos
$p57H19^{+/-}$ X C57BL/6	24		170
		p57H19	99
		+/+	71
C57BL/6 X $p57H19^{+/-}$	6		52
		p57H19	12
		+/+	14
$p57H19^{+/-}$ X $Igf2^{+/-}$	10		75
		p57H19	14
		$p57H19; Igf2^{+/-}$	26
		$Igf2^{+/-}$	15
		+/+	20
$H19\Delta13^{+/-}$ X C57BL/6	4		26
		$H19\Delta13^{+/-}$	12
		+/+	14
$p57^{Kip2+/-}$ X C57BL/6	4		30
		$p57^{Kip2+/-}$	16
		+/+	14
$p57^{Kip2+/-}$ X $Igf2^{+/-}$	2		22
		$p57^{Kip2+/-}$	2
		$Igf2^{+/-}$	7
		$p57^{Kip2+/-};Igf2^{+/-}$	6
		+/+	7

Embryos of the crosses indicated were genotyped between 12-18.5 d.p.c. Female parent indicated first.

Table 2 *Growth of p57H19 mutant embryos*

Tissue	P57H19 (BL/6) n = 20	p57H19 (BL/6-129) n = 8	$Igf2^{+/-}$ (BL/6-129) n = 10	p57H19; $Igf1^{+/-}$ (BL/6-129) n = 19
Embryo	1.04	1.06	0.58*	0.93
Placenta	1.94*	1.53*	0.55*	1.0
Tongue	1.22*	1.11	0.86	1.00
Heart	1.03	1.17	1.03	1.16
Kidney	1.13	N.D.	N.D.	N.D.
Liver	0.83	0.98	0.75	0.89

The wet weights of embryos and internal organs of the genotypes indicated are expressed as a fraction of that of wild type littermates. The p57H19(BL/6) amimals were derived from crosses to C57BL/6 males; all others were derived from a $p57H19^{+/-}$ x $Igf^{+/-}$ cross. (N.D.). Not determined. *$P < 0.05$.

Table 3 *Summary of phenotypes in BWS animal models*

Phenotypes	Percent Observence		
	BWS	p57H19	+/+
Macroglossia	95	60(12/20)	11(1/9)
Adrenal defects cytomegaly	94	0	0
" cysts	69	0	0
Placentomegaly	92	96(27/28)	13(2/15)
Visceromegaly hepatomegaly	85	16(3/19)	11(1/9)
" nephromegaly	69	40(8/20)	13(1/8)
" cardiomegaly	N.D.	15(3/20)	13(1/8)
Somatic overgrowth	60	39.2(11/28)	13(2/15)
Renal dysplasia	59	61.5(8/13)	0
Abdominal wall defects omphalocele	60	4(1/23)	0
" " umbilical hernia	32	48(11/23)	0
Cleft palate	7.1	26(6/23)	0
Subcutaneous cleft palate	20	N.D.	N.D.
Cardiac defects	20	39(5/13)	0
Polydactyly	5.5	30(7/23)	0

The frequency of the phenotypes is expressed as a percentage of all individuals examined. The BSW data are adapted from Eggenschwiler et al. (1997). The p57H19 and +/+ data are taken from 18 d.p.c. p57H19 x C57BL/6) litters. (N.D.) Not determined.

Table 4. *Effect of reducing IGFII in p57H19 mice*

	Genotype	
Phenotype	p57H19	p57H19; Igf2
Postaxial polydactyl	50(4/8)	0(0/15)
Cleft palate	12.5(1/8)	20(3/15)
Omphalocele	37.5 (3/8)	20(3/15)
Umbilical hernia	25(2/8)	27(4/15)

The frequency of the phenotypes in 18 d.p.c. fetuses are expressed as a percentage of all individuals examined.

Personnel

Stephen J. Elledge
J. Wade Harper
Pumin Zhang
Calvin Wong

Publications

Caspary, T., Cleary, M.A., Perlman, E.J., Zhang, P., Elledge, S.J., and S.M. Tilghman. (1999) Oppositely imprinted genes p57(Kip2) and igf2 interact in a mouse model for Beckwith-Wiedemann syndrome. *Genes Dev.* 13:3115-24.

Zhang, P., Wang, C., Liu, D., Finegold, M., Harper, J. W., and S. J. Elledge. (1999). p21^{CIP1} and p57^{KIP2} control the development of skeletal muscle at myogenin step. *Genes Dev.* 13, 213-224.

Lahoz, E. G., Liegeois, N. J., **Zhang, P.**, Engelman, J. A, Horner, J., Silverman, J., Burde, R., Roussel, M. F., Sherr, C. J., Elledge, S. J, and R. A. DePinho. (1999). Cyclin D- and E-dependent kinases and the p57^{KIP2} inhibitor: cooperative interactions in vivo. *Mol. Cell. Biol.* 19:353-363.

Zhang, P., Wong, C., DePinho, R.A., Harper, J.W., and Elledge, S. J.(1998). Cooperation between the *Cdk* Inhibitors *p27^{KIP1}* and *p57^{KIP2}* in the control of tissue growth and development. *Genes Dev.* (in press).

Elledge, S.J. (1998) Mitotic Arrest: Mad2 prevents sleepy from waking-up the APC. *Science* 279: 999-1000.

Harper, J.W. and Elledge, S.J. (1998) The role of Cdk7 in Cdk activation; a retro-retrospective. *Genes and Dev.* 12: 285-289.

Zhang, P., Liegeois, N.J., Wong, C., Hou, H., Finegold, M. Silverman, A., Harper, J.W., DePinho, R.A., and Elledge, S. J.(1997). Altered cell differentiation and proliferation in mice lacking p57^{KIP2} indicates a role in Beckwith-Wiedeman Syndrome. *Nature* 387:151-158.

Dynlacht, B.D., Ngwu, C., Winston, J., Swindell, E.C., Elledge, S.J., Harlow, E., and Harper, J.W. (1996) Purification and Analysis of CIP/KIP Proteins, *Methods in Enzymology* 283:230-244.

Elledge, S.J., Winston, J., and Harper, J.W. (1996) A question of Balance: The roles of cyclin kinase inhibitors in development and tumorigenesis.*Trends in Cell Biology* 6, 388-392.

Lee, P., DeBaun, M., Randhawa, G., Reichard, B., Elledge, S.J., and Feinberg, A.P. (1997) Low frequency of p57^{KIP2} mutations in Beckwith-Wiedeman Syndrome. *Amer. J. Human Genet.* 61:304-309.

Elledge, S.J. (1996) Cell Cycle Checkpoints: Preventing an Identity Crisis. *Science* 274:1664-1672.